IDENTIFICATION AND CHARACTERIZATION OF SPECIALIZED ANTIGEN PRESENTING CELLS IN RAINBOW TROUT

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IDENTIFICATION AND CHARACTERIZATION OF SPECIALIZED ANTIGEN PRESENTING CELLS IN RAINBOW TROUT

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As the oldest vertebrates to possess intact innate and adaptive immune systems fish are the ideal models to study evolution of adaptive immunity. With the advent of adaptive immunity came a new challenge: the coordination of temporally and spatially diverse responses to pathogens. In mammals, dendritic cells (DCs), as a result of their distinctive activation program and specialization in antigen presentation, function as a link between innate and adaptive immunity. The question as to whether dendritic cells arose concurrently with adaptive immunity or at a later date is unknown. It is possible that antigen presentation in fish is primarily accomplished by another cell type or types.

The goal of my research was to identify the functional equivalent of mammalian dendritic cells in fish. In order to do this, significant numbers of cells were needed to enable functional characterization of presumptive dendritic cells. Dendritic cells are a rare cell type and in order to generate adequate numbers of cells for in depth analysis, protocols have been developed in mammals to culture dendritic cells. My approach to the problem, therefore, was to adapt these mammalian protocols to rainbow trout. Development of a hematopoietic culture system in rainbow trout, based on bone marrow derived DC cultures in mammals, generated DC-like cells (DCLCs) that were then characterized using criteria for classification of mammalian DCs.

DCLCs resembled mammalian DCs in their expression of CD83 and MHCII, phagocytic capacity, and response to TLR-ligands; however, the most remarkable similarity was their ability to stimulate potent primary MLRs, more vigorous than those obtained with either macrophages or B-cells as stimulators. This evidence for a specialized antigen presenting cell type in rainbow trout has implications for fish vaccine development as well as comparative studies with mammals to elucidate the origins of adaptive immunity.

BIOGRAPHICAL SKETCH

Elizabeth Bassity, better known as Lizzy, was born in Gouverneur, NY on January 6, 1978. She was one of four girls who always got along well and never fought over the bathroom. She spent her formative years in the "north country", on the St. Lawrence River. Her parents always placed great importance on education and opportunities to broaden their childrens' horizons. She attended the Campus Learning Center lab school at SUNY Potsdam, and later also attended high school in Potsdam, where her superlative Biology teacher managed to captivate her all-encompassing enthusiasm for science and secure a particular attachment to the field of Biology.

For college Lizzy headed off to the big city. She attended the University of Chicago, which she chose based on their core curriculum. The core allowed her to indulge in liberal arts and strong sciences in pursuit of her renaissance-woman ideal. She found the adage that the U of C is "where fun comes to die" had some truth, as she was forced to take a leave of absence due to illness, but eventually, still plagued by fatigue, graduated on time. Her first-hand experience with the ravages of infectious disease led Lizzy to her enduring interest in Immunology, or as she likes to think of it: the war between good and evil, where often the most difficult problem is trying to tell the difference.

Along the way Lizzy worked in a research lab as an undergrad and spent a summer as an intern at the NIH. After graduating with general honors, Lizzy moved to Boston, MA where she worked as a lab tech for two years at Tufts University and Boston Medical Center. Her experience working as a tech led her to the conclusion that despite her vows never to go back to school, graduate education would afford her the

opportunities she desired to learn to her heart's content, unfettered by (other peoples') endless lab chores.

Things rarely end up as we expect them to, and graduate school was no exception for Lizzy. Her plan was to work in an infectious disease model, but instead she joined a lab that did not do any overt Immunology. There she conceived of her own project, a search for antigen presenting cells in fish, with the hope that their identification would (some day) lead to the development of fish as a useful disease model.

This dissertation is dedicated to Bunky.

I wish you were here.

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Introduction

1. Fish Immunology

Osteichthyes, the bony fishes, comprises the largest number of extant species in a vertebrate class; upwards of 25,000 species. The teleosts represent the largest subgroup; virtually all living fishes are within this class. Fish are the earliest group to have intact innate and adaptive immune systems and as such are an excellent model for the study of the evolution of adaptive immunity. The immunology of several species has been studied extensively, most notably rainbow trout (*Oncorhynchus mykiss*), and channel catfish (*Ictalurus punctatus*), which have been used for functional studies; and zebrafish (*Danio rerio*) and Fugu (*Fugu rubripes*) used extensively in genomics studies. The complete genomes of Fugu and zebrafish have been sequenced. The genome of Fugu is of particular interest because of its small size (one of the shortest vertebrate genomes at 400Mb), a result of the absence of long repeat sequences found in other vertebrates.

In the realm of basic research, a greater understanding of fish immunology has applications in several areas in addition to comparative genomics. Because fish are the first vertebrates to mount both innate and adaptive immune responses, the study of fish immunology will reveal the evolutionary origins of adaptive immunity. It is unknown whether DCs, which act as the link between innate and adaptive immune responses in mammals, co-evolved with adaptive responses or if another cell type(s) performed this function early in vertebrate history. The identification of DCs or a similar cell type in fish would go a long way towards answering this question. The determination of the evolutionary origin of DCs will help address the pertinacious questions in mammalian immunology as to the plasticity and/or heterogeneity of DCs. Furthermore, advances in fish immunology will allow fish to be used as an immunologic and disease model. Evolutionarily distant species have not traditionally been used as immunological

models; however, information central to our current understanding of mammalian immunology has resulted from the study of such species. The Toll gene was discovered in the invertebrate *Drosophila* in 1985, and was subsequently shown to have a dual role in development and the innate immune response (1, 2). This system is so ancient that homologues of the *Drosophila* genes in the Toll pathway can be found in plants where they also play a role in disease resistance (3). Toll-like receptors (TLRs) were consequently identified in humans in 1997 and have since been demonstrated to be a major mode of identification of pathogens by the mammalian immune system (4). This important discovery, crucial to our current understanding of immunology, was based on work done in an invertebrate. Clearly, study of our ancient ancestors is relevant to our understanding of mammalian immunology.

Fish are excellent model organisms for a variety of reasons and their strengths differ from those of popular models such as the mouse. Having a well developed alternate model with different strengths allows the use of different approaches and techniques. In particular, forward genetics and transgenesis have proved to be efficacious in fish. Also, it seems that fish more closely replicate the human disease state than mouse models in some cases (5). Additionally, cutaneous immunity in fish appears to be mediated by a mucosal mechanism, which may simplify study of mucosal immunity by allowing experiments on an extensive external surface (6). Because of the differences from the mammalian immune system, we are likely to learn different information from fish than mice or other models. Coming from a different perspective often results in new discoveries and innovations. By providing an alternative model, there is the opportunity to try new approaches and methods.

Besides the usefulness of fish in comparative studies, fish immunology is of great importance on a practical level. Aquaculture is the fastest growing animal food production sector with total world production valued at US $70.3 billion in 2004 (7). With the depletion of wild stocks, the market is increasingly reliant on aquaculture for production of food-fish. Aquaculture practices typically involve dense culture methods that can result in high losses from infectious disease estimated to be worth millions of dollars each year. In addition, zebrafish are an important resource as a model organism that has been invested in substantially by many, including the NIH. A greater understanding of fish immunology will prove inestimable in maintaining the health of these valuable stocks (8). There are vaccines available commercially for several fish diseases; however, a more complete understanding of how the adaptive immune response is mounted would be invaluable in the design of new and more effective vaccines and treatments.

Because of the difficulties in using classical immunological methods in fish, genomics serves as an important alternative. Genomics is the study of organisms at the level of the DNA sequence. In order to extract information from DNA, a comparative approach to known genes and regulatory sequences is used. To efficiently compare DNA sequences to the large databases of known sequences that are available, programs that can compare DNA sequence on the local and global level are used. BLAST (Basic Local Alignment Search Tools) is a set of sequence comparison algorithms that makes pair-wise comparisons on the local and global level resulting in a score that indicates the quality of the alignment. The expectation value (E value), commonly used to judge alignments, is the number of alignments with a score equal to or better than the raw score for that alignment that are expected to occur in a database by chance. Raw score is based on identities, mismatches and gaps in the alignment.

The lower the E value the more significant the score. In the comparison between fish and mammalian genomes, the evolutionary distance results in higher E values than are typically expected even in homologous regions. Open reading frames for homologous genes can have identities with mammals as low as 20-30%. Conserved regions within these genes tend to have higher identity. Local alignments of conserved regions (as identified by comparisons within mammals and to other species for which there is information available) can facilitate the identification of homologs. Additionally, regulatory regions and synteny can be informative in the identification of homologs.

The sequencing of the first two vertebrate genomes, the human genome (first draft available in 2000) and Fugu (draft finished in 2002), began the era of comparative genomics. Since then, comparative genomics has been a tool of choice in studying fish immunology. This method allows researchers to leverage the extensive knowledge we have of mammalian immunology for use in fish, where tools and resources are considerably more limited. The success of this approach thus far demonstrates the conserved nature of the immune system as a whole at the genetic level. The identification in fish of the basic components of the innate and adaptive immune systems, both on the cellular and molecular level, suggests that function is also highly conserved. In addition, comparative genomics also gives us insights into mammals. For example, large numbers of predicted genes, not previously identified in humans, have come to light by comparison with the Fugu genome.

2. Innate Immunity

Innate immunity consists of physical barriers, and germ-line encoded defense mechanisms that have been effective against pathogens for eons. Because of the rapid evolution of pathogens, innate immunity targets conserved molecules and pathways

that have not changed substantially over long evolutionary periods. Molecules recognized by the innate immune system are typically called pathogen associated molecular patterns (PAMPs) and are recognized by pattern recognition receptors (PRRs).

The toll-like receptors (TLRs) are one such family of PRRs that, as mentioned, are extremely highly conserved and can be traced back to plants. The first TLR homolog identified in fish was found in the goldfish (9). TLR-21 and -22, unique to fish, were subsequently identified in Fugu (10). Currently, 19 putative TLRs have been identified in zebrafish as well as the TIR domain adaptors MyD88, MAL, TIR-domain containing adaptor inducing INF-β (TRIP), TRIF-related adaptor protein (TRAM), and sterile α and HEAT-Armadillo motifs (SARM) (11, 12). In rainbow trout, two TLR-5 orthologs have been identified: a membrane bound and a secreted form (13). Bacterial flagellin has been identified as the ligand. In Fugu, TLR-22 and TLR-3 have been established to recognize double stranded RNA (dsRNA) (14). TLR-22 is expressed on the surface of cells and recognizes long dsRNA, while TLR-3 is expressed intracellularly and recognizes short dsRNA. Although up-regulation of TLRs in response to viral and bacterial pathogens has been observed, specificity of other TLRs is yet to be determined (12, 15).

Fish possess a variety of other innate receptors including lectins (16-19), scavenger receptors (20-22), complement proteins (23-25), and Nod-like receptors (26). In addition, unique to fish, are the novel immune type receptors (NITRs) (27-30), the novel immunoglobulin-like receptors (NILTs) (31, 32), and non-specific cytotoxic cell receptor protein (33). The fish complement system is highly developed. In fish, factor B functions in the alternative and the classical pathway (34). Classical pathway titers

in fish and mammals are similar, however, alternative pathway titers are 5-10 times greater in fish (35). Fish have multiple isoforms of many of the complement factors, and it seems that, as a result, they may serve more diverse functions than in mammals (36, 37).

Antimicrobial peptides (AMPs) can be found in fish skin, gut, gills, in the granules of granulocytes (38), in red blood cells (39), liver (40), leukocytes (41), and plasma (42). These peptides can be derived from hemoglobin (43), ribosomal proteins (44), or histones (45-49) (or other chromosome associated proteins (50)). The plethora of AMPs present in fish could potentially be used against mammalian bacterial pathogens in place of traditional antibiotics, which are rapidly becoming obsolete as a result of acquired resistance. Antimicrobial peptides from fish (and frogs) have been described to inactivate viruses (51, 52), positioning them as promising new candidates for viral treatment in humans. Indeed, a frog antimicrobial peptide has been shown to potently inactive human immunodeficiency virus (53).

A variety of cells that effect the same response as mammalian granulocytes are present in fish, however, debate exists as to their correspondence to the particular types present in mammals. Although there is variance among species, neutrophilic/heterophilic, eosinophilic, and basophilic granulocyte type cells are present in fish (54). High levels of MHCIIα transcripts have been detected in acidophilic granulocytes suggesting they may be APCs in fish, however, in the same study all leukocyte populations examined were found to express MHCIIα transcripts (55). Rainbow trout possess eosinophilic granule cells (EGC) that are suggested to be homologs of mammalian mast cells, or are excluded from that possibility, depending on criteria used (56-60). These cells are generally considered restricted to intestine,

with one report of ECG in gill and skin in addition to gut (58). The main granulocyte in rainbow trout is the neutrophil. Rainbow trout neutrophils have irregularly shaped nuclei (not unlike trout macrophages (61)) and oval, electron dense granules in their cytoplasm (62). Functionally, neutrophils are phagocytic, produce nitric oxide synthase (involved in killing of intracellular pathogens), and large amounts of superoxide anion (a measure of respiratory burst activity). Following intraperitoneal (i.p.) injection of adjuvant or killed bacteria, recruitment of neutrophils peaks in 24-48 hours (63), suggesting their role early in inflammation as described for mammalian neutrophils.

Fish have macrophages that appear similar to mammals in structure and function. They are adherent, phagocytic cells that undergo respiratory burst and produce nitric oxide. There is evidence that classical as well as alternative activation of macrophages occurs in fish (64). Methods for *in vitro* culture of fish macrophages have been developed (65, 66). These methods involve isolation of mononuclear cells followed by adherence and removal of non-adherent cells. Stafford et al isolated cells using a 51% percoll gradient and added conditioned medium (from mitogen stimulated head kidney leukocytes) to generate monocytes in trout, with no evidence of a mature macrophage cell type. However, in goldfish they report that macrophages can differentiate either from monocytes or directly from hematopoietic precursors, using the same method (65). Monocytes were characterized by forward side scatter profile, morphology, generation of reactive oxygen intermediates (ROIs), and the inability to generate reactive nitrogen intermediates (RNIs). Mackenzie at al. use Ficoll-Histopaque to isolate monocytes from brook trout and brown trout. They, like Stafford et al, describe the same three populations in their cultures; however, they identify one of these as differentiated macrophages that are the only cell type present by day six of culture.

They describe monocytes as smaller and poorly phagocytic compared to the *in vitro* differentiated macrophages. TNF-alpha mRNA expression was induced in both cell types in response to LPS; however, the magnitude of the response differed greatly; macrophage transcript levels were more than 6 times greater than monocyte levels. In subsequent experiments in rainbow trout, unfractionated head kidney cells were plated on lysine coated plates and allowed to adhere for twenty-four hours. Non-adherent cells were washed off and the remaining cells were cultured for four additional days, resulting in macrophages or mononuclear phagocytes (67, 68). In catfish, seminal studies on antigen presentation used monocytes/macrophages (no distinction made). These cells are described as peripheral blood leukocytes that adhere to baby hamster kidney cell microexudate-coated surfaces (presumed to be fibronectin) or Sephadex G-10 (69).

LPS stimulated rainbow trout macrophages express a range of immune-related genes including CD83, CD209, MHC I and II proteins, lectins, cytokines and chemokines, and cytokine and chemokine receptors (67). CD83 has since been reported as an activation marker for fish macrophages (70). Using TNF as a read-out, rainbow trout mononuclear phagocytes are activated by zymosan, and muramyl dipeptide (MDP) in addition to LPS (68). Macrophages in culture express the transcription factor PU.1, a regulatory protein involved in myelopoiesis in mammals (71).

Long term monocyte/macrophage cell lines in catfish arose spontaneously in culture resulting in cells that are phagocytic, secrete IL-1 in response to LPS, stimulate the MLR, and resemble mammalian monocytes/macrophages (72). The rainbow trout macrophage-like cell line, RTS-11, consists of two cell types, a small round precursor population and a large, granular, phagocytic, macrophage-like population (73).

Transcriptional profiling revealed that rIL-1 treatment of RTS-11 cells up-regulated genes involved in the acute phase response, while rIFN-γ up-regulated the major histocompatibility class I (MHC class I) pathway (74).

Isolation of resident macrophages from rainbow trout peritoneal cavity has been reported (61, 75, 76). The percent of recovered cells that are identified as macrophages in un-elicited lavage fluid range from 33% (61) to 100% (75). Resident macrophages are phagocytic, non-specific esterase and acid phosphatase positive, and myeloperoxidase negative.

In the absence of good markers, distinguishing monocytes from macrophages has been problematic in fish, particularly in culture. The identification of macrophage colony stimulating factor (M-CSF), its receptor (M-CSFR), and subsequent generation of an anti-M-CSFR antibody in Gilthead seabream have allowed a more definitive identification of macrophages in that species (77). This antibody specifically stains the adherent cells from head kidney, and in tissue sections, stains cells in the head kidney, spleen, thymus, gills, and liver, but not intestine. The absence of M-CSFR mRNA expression, coupled with CD83 expression in the phagocytic Atlantic salmon TO cell line has prompted comparisons of these cells to mammalian dendritic cells (78).

In addition to traditional macrophages, fish possess melanomacrophages, which are pigmented macrophages that aggregate in lymphoid tissue forming melanomacrophage centers (MCCs) (54). Based on the retention of antigen and close association with Ig-secreting cells seen in MCCs, it has been suggested that these are equivalent to germinal centers, but others argue that they are merely scavengers in lymphoid organs because of the lack of reticular cells in MCCs (79). The specific

staining of melanomacrophages with the follicular dendritic cell antibody CNA-42 suggests they may indeed be forerunners of follicular DCs (80). An Atlantic salmon cell line (SHK-1) that produces melanin (a characteristic of melanomacrophages) reportedly expresses CD83 mRNA transcripts (81). Proteomics analysis of rIFNγ treated SHK-1 cells demonstrated increased expression of a complement component, MHC class I, and MX (82). Type I IFN mRNA transcripts were also detected as a result of IFNγ addition. More studies are needed to clarify the role these cells may play in an immune response.

Intriguingly, fish thrombocytes (cellular homologs of mammalian platelets) have been found to express MHC class Ia components, IL 1β, TNFα, TGF-β, interleukin receptor common γ chain, C X C and CC chemokines (83). More recent data show that surface expression of MHC class II on thrombocytes is not consistent from individual to individual in catfish (84). It must be mentioned that the antigen recognized by the thrombocyte-specific antibody in this study has not been completely characterized; it may recognize other cell populations under certain conditions. However, these data do suggest these cells may play a role in the fish immune system.

A novel immune cell type, nonspecific cytotoxic cells (NCCs), has been described in fish (54). These cells are NK-like cells that are distinguished by the expression of novel immune type receptors (NITRs). NITRs have extracellular variable (V) and V-like C2 domains, a transmembrane region, and a cytoplasmic tail– typically with an immunoreceptor tyrosine-based inhibition motif (ITIM) (28). There is a close relationship between the NITR V domains and those found in Ig and TCR. These cells display non-specific cytotoxic activity, but have also been implicated in recognition of

allogeneic cells, through a “missing self” mechanism similar to that of mammalian NK cells (85).

3. Adaptive Immunity

The classic method utilized in immunology to identify particular cells, based on antibody binding to specific surface molecules, has thus far proved ineffective in fish. The reason for this is not completely clear. It is thought that the heavily glycosylated surface of fish cells results in production of antibodies that bind to the glycosylated surface of all fish cells, rather than the specific antigen target (79).

Because of its high density on the cell surface and methods available to purify immunoglobulin (Ig), a monoclonal antibody to trout immunoglobulin M (IgM) was developed and characterized in 1983 (86). No other B-cell surface markers are available. Fish B-cells are small lymphocytes that produce specific antibodies (both membrane bound and secreted) in response to antigen. The number of antibody isotypes was thought to be restricted compared to mammals; however, recent evidence suggests that fish possess additional classes of Ig that are altogether different from those in mammals. Like mammals, fish possess IgM and IgD, but unlike mammals IgZ (87), IgT (88), a chimeric immunoglobulin (IgM-IgZ) in carp (89), and a novel Ig heavy chain in Fugu (90) have been described. IgM, in a tetrameric form, is the dominant class expressed, with expression of IgZ seen primarily early in development. The roles of other classes of Ig have not been elucidated. It was recently reported that a polymeric immunoglobulin receptor was involved in transport of tetrameric IgM to the mucus of the skin in Fugu (91), much the same as antibody is transported in the gut of mammals. Isotype switching is thought to be absent, although IgM in trout is found in different oxidation states, which has been hypothesized to serve a similar

function (92). Fish B-cells have not been observed to form germinal centers or to undergo somatic hypermutation; however, it has been demonstrated that fish activation-induced cytidine deaminase (AID) can catalyze class switch recombination and somatic hypermutation when expressed in mammalian cells (93, 94). Other possible mechanisms of affinity maturation are under investigation (95). Fish B cells proliferate in response to the typical mammalian B cell mitogen LPS (albeit at much higher concentrations than in mammals) (96). B-cell antibody production specific for T-dependent and T-independent antigens has been demonstrated; both require the presence of accessory cells (97, 98). B-cells of rainbow trout have been shown to be phagocytic and capable of killing ingested bacteria (99). The authors suggest that this is evidence that lymphocytes evolved from an ancestral phagocytic cell type (i.e. macrophages).

All four T cell receptor (TCR) chains (α,β,γ, and δ) have been identified in Japanese flounder (100). CD8 and CD4 have been identified in several species (101-106). The recent discovery of CD4 and family members in trout has suggested that CD4 arose in fish through a duplication event within a related gene (CD4REL), as CD4REL contains two Ig domains, while trout CD4, like mammalian CD4, contains four (107). Predicted protein sequences, when compared with mammals, showed differences within the region involved in MHC class II binding, raising questions as to whether interaction between the two molecules occurs in the same manner in mammals and fish. On the other hand, evidence suggests that T-cell signaling is conserved from fish to mammals (108). Molecules involved in APC/T-cell communication including CD3 (TCR co-receptor), CD40, MHC class I and MHC class II have been identified (109-111). The costimulatory receptors CD28 and CTLA-4 have been described in trout, however, CTLA-4 does not have the signaling motifs of its mammalian counterpart

(112, 113). Cell mediated toxicity against allogeneic targets has been demonstrated in several species (114-116), as well as graft rejection (117-120), and delayed type hypersensitivity (121). Fish T cells respond to typical mammalian T cell mitogens such as ConA and PHA (122). In response to mitogenic stimuli, trout T-cells produce an interleukin-2 like factor (123).

Key non-cellular components of the immune system have been identified in various fishes. Cytokines including IFNγ, IL-1β, IL-2, IL-6, TGF-β, TNF-α, IFN-α, IFN-β, IL-8, IL-10, IL-11, IL-12, IL-15, IL-18, IL-21, IL-22, and IL-26 have been identified (124-128). Numerous CC and CXC chemokines have been described (129). Despite significant differences at the genetic level, bioactivity across species boundaries of several cytokines has been demonstrated. Human IL-1 potentiates the response of catfish lymphocytes to ConA (130). Supernatant from activated carp macrophages and neutrophilic granulocytes stimulates proliferation of the murine IL-1 dependent cell line D10(N4)M (131). This activity is abolished by antibodies to human IL-1. Recombinant human TNF-α synergizes with trout MAF to elevate respiratory burst in trout macrophages and with mitogens to increase proliferation in leukocytes (132, 133). Supernatants from LPS-treated trout macrophages biologically cross-react with mammalian cells (134). This interaction is inhibited by anti-mouse TNF-α. Bovine TGF-β increases respiratory burst in trout macrophages at low doses, but decreases respiratory burst as dose increases (135). Anti-mouse IL-2 antibody binds to culture supernatant fractions of mitogen activated trout T-cells in Western blots, and inhibits proliferation induced by conditioned culture medium (123).

With the knowledge of fish immunology we have so far, it seems there are more similarities than differences from mammalian immunology. However, there are two

differences of significant import. Fish do not have bone marrow; the head kidney, and in some cases also spleen are the bone marrow equivalent(s) in fish. Fish do not have lymph nodes; however, their spleen architecture is similar to that in mammals, with distinct T-cell and B-cell zones. Because of this it is thought that antigen presentation occurs in the spleen and also possibly head kidney in fish, but this has yet to be demonstrated. Formation of germinal centers has not been observed in fish, but there is evidence that antibody secreting plasma cells are largely restricted to the anterior kidney (136).

4. Antigen Presentation

Monocytes, the precursor to macrophages and DCs in mammals, have been described as the accessory cell type in fish necessary for antigen presentation to occur. Given the overall homology between the fish and mammalian immune systems, it is unlikely that antigen presentation occurs in a substantially different way in fish. The limited functional experiments that have been done demonstrate a similar process in fish.

Mixed leukocyte reactions (MLRs), the gold standard assay for antigen presentation, have been demonstrated in several species of fish including rainbow trout (137-140). Experiments examining the mechanism of antigen presentation in fish, however, are not as widespread. The seminal studies were conducted in channel catfish by the laboratory of William Clem. Clem first established a role for "accessory cells" or APCs by determining that Ig-negative lymphocyte (presumably T-cells) responses to LPS or ConA required the presence of monocytes/macrophages (described above) (69). It was also observed that supernatants from monocytes/macrophages were sufficient to fulfill this accessory role, which was attributed to the presence of IL-1. He went on to further demonstrate that monocytes/macrophages, B-cells, and to a

lesser extent, Ig-negative lymphocytes, could stimulate the MLR, whereas Ig-negative lymphocytes were the only cells capable of acting as responders (141). The number of monocytes/macrophages that could be isolated was limited, preventing a direct comparison with B-cells, although monocytes/macrophages appeared to be better stimulators. An interesting quirk that Clem reported was that MLRs were only seen when autologous monocytes/macrophages were present in the responder population. It was long thought that MLRs were stimulated directly by interactions between stimulator DCs and allogeneic responder T-cells. Our current understanding of the MLR, however, indicates that while this direct stimulation does occur, transfer of MHC molecules from stimulator DCs to responder DCs is the primary mode of activation of responders, as evidenced by the significantly reduced reaction with the depletion of responder DCs (142). In fact, responder DCs can acquire MHC from cell-free stimulator supernatants and present it to responder T-cells. It seems that this ability to transfer MHC molecules is restricted to DCs (142), another specialization that contributes to their professional APC function (143). This new understanding of MLRs illuminates the early findings in catfish. Clem's observation can now be explained if the monocytes/macrophages have this specialized ability to acquire MHC molecules from stimulator cells that can then be presented to responder cells.

The cell-free presentation of antigen was also demonstrated by Clem with the isolation of crude membrane preparations from antigen pulsed APCs that were capable of stimulating peripheral blood lymphocytes (PBLs) (144). In the same set of experiments, radioactive labeling of antigen was used to trace the movement of antigen through distinct cellular fractions. In the first hour of incubation, antigen was primarily associated with the cell membrane; association with the endosomal/lysosomal fraction peaked at 3 hours, and then the radioactivity

redistributed out to the cell membrane after 5 hours of incubation. This antigen path is consistent with the processing and presentation of class II antigens in mammals. Antigen is taken up, often a receptor mediated process, and trafficked to lysosomes where antigen is digested into peptides. Meanwhile MHC class II complexed to the MHC class II invariant chain is transported from the ER to the endosomal compartment. The invariant chain is digested, leaving the MHC class II associated invariant chain peptide (CLIP) in the MHC class II groove. An MHC class II-like molecule, called HLA-DM in humans, mediates the exchange of the CLIP peptide by binding the CLIP itself with higher affinity, leaving the MHC class II to bind the antigen peptides present in the endosome (a more stable interaction than with CLIP). The peptide-MHC complex then continues its transport to the cell membrane. Interestingly, two invariant chain genes have been found in fish (145, 146). Due to their expression pattern that parallels that of MHC class II, they have been implicated in antigen presentation, but their roles have not been elucidated.

The ablation of acidic endosomes or inhibition of proteases diminishes antigen redistribution to the surface, demonstrating a dependency on these compartments, and presumably the digestion into peptides that occurs there (138). The elimination of this step, which is presumably necessary for the formation of peptide-MHC complexes, then prevents the antigen from trafficking to the cell membrane. This dependence on lysosomal processing could be bypassed by pulsing the APCs with antigen peptide (144).

Clem also examined the requirements for antigen presentation in secondary responses. B-cells or monocytes/macrophages pulsed with antigen and fixed could stimulate proliferation and specific antibody production in syngeneic PBLs from previously

vaccinated fish (138). This was genetically restricted (presumably by MHC compatibility) as the use of allogeneic PBLs resulted in an antigen non-specific MLR with no antibodies produced. Autologous monocytes not pulsed with antigen did not elicit responses. Like the primary responses described above, dependence on lysosomal processing of the antigen was again observed. All of these data put together indicate that processing of antigen and subsequent presentation on the cell surface in fish occurs in a strikingly similar manner to mammals.

5. Dendritic Cells

DCs are professional antigen presenting cells of myeloid and lymphoid origin. They were first described by Ralph Steinman in the 1970's as non-phagocytic, adherent cells of low buoyant density with a stellate shape (147-150). This distinctive shape first distinguished these cells from macrophages, which are typically round, strongly adherent cells. Functional studies of these rare, oddly shaped cells led to the discovery of a cell type that has dominated recent immunologic history. Functionally, the superior ability to stimulate the MLR (up to 100 times more stimulatory) set DCs apart from macrophages and other APCs (151). The first identification of DCs was in the white pulp of the mouse spleen. Steinman determined that DCs were bone marrow derived and underwent rapid turnover in the secondary lymphoid tissue. Since then, DC biology has become considerably more complex.

In humans, two primary populations of DCs have been described: plasmacytoid DCs (pDCs) and myeloid or conventional DCs (152, 153). Plasmacytoid DCs are named for their resemblance to plasma cells and can be of myeloid or lymphoid origin (154). Differentiation of these cells from CD34+ progenitor cells occurs in the presence of Flt-3 ligand and their lifespan is extended by the presence of IL-3 (155). pDCs are

generally less efficient than myeloid DCs at antigen capture and presentation (156). A distinct function of these cells is their ability to produce large amounts of type I interferon in response to viruses (155). Preferential expression of TLR 7 and TLR 9 mediate this unique function. Myeloid DCs express CD11c+, CD11b+, CD33+, and CD13+ and express TLRs -1, -2, -3, -4, -5, -7, and -8. Conventional DCs can be further divided into monocyte derived DCs, interstitial DCs (iDCs) and Langerhans DCs (LCs) (157). DCs are present in the dermis and interstitium and LCs are found in the skin and mucosa, both in immature form. iDCs and LCs are potent APCs; however, the interstitial DCs alone are capable of inducing the differentiation of naïve B cells into plasma cells. A distinctive attribute of LCs is the presence of Birbeck granules in their cytoplasm. Birbeck granules are rod or racquet shaped electron dense vesicles that have distinct "zippered" striations. The formation of Birbeck granules is dependent on Langerin (CD207, a C-type lectin), which mediates uptake (into Birbeck granules) and processing of non-peptide antigens (158). Both populations can be derived from CD34+ bone marrow cells or CD11c+ blood precursors. *In vitro*, the presence of GM-CSF or TNFα +/- IL-4 is required to generate monocytes and iDCs, LCs additionally require TGF-β.

DC subsets pertaining to human cDCs and pDCs have been identified in mice, but the subsets of DCs have traditionally been categorized as CD8+, CD8-, or LCs (also CD8–). CD11c is the best marker for murine DCs, as all DC subsets are CD11c+. A population suggested to be equivalent to human pDCs has been described as CD11c+, B220+, and Gr1+ (159). These cells are plasmacytoid and produce large amounts of IFN-α in response to virus.

The essential and unique function of DCs is the induction of the primary immune response. According to the current "danger signal" hypothesis, proposed by Polly Matzinger, this process is initiated by immature DCs in the periphery encountering antigen in the presence of danger signals (160). TLRs, C-type lectins and other PRRs recognize PAMPs and transduce a signal that triggers DC maturation. Maturation occurs en route to the draining lymph nodes and consists of the secretion of cytokines and up-regulation of MHC class II and costimulatory molecules. The migration of DCs is accomplished through the downregulation of chemokine receptors such as CCR6 that mediate recruitment to sites of inflammation, and the up-regulation of CCR7, thereby gaining responsiveness to chemokines produced in lymph nodes, resulting in homing to lymph nodes. DCs migrate to the T-cell rich zones of the spleen and lymph nodes where interactions with T-cells occur. Along with TCR ligation by peptide-loaded MHC, costimulatory molecules such as CD40, CD80, and CD86 interact with CD40L and CD28 on T-cells. CD83 is up-regulated with costimulatory molecules on DCs, but its role is not completely clear. It has been reported to be involved in lymphocyte development and activation (161-163) and regulation of MHC class II surface expression (164). Effective stimulatory DC-T-cell interaction leads to the up-regulation of CD40L on T-cells and ligation of CD40 on DCs leading to IL-12 release. CD40L expression on T-cells provides the required activation signal to B-cells.

Depending on the type and duration of stimulus and antigen load, DCs can polarize the T-cell response towards a Th1, Th2, or Th17 response. It was initially reported that pDC-mediated activation resulted in a type 2 response and myeloid DCs resulted predominately in a type 1 response, but it now seems clear that this is an over-simplification; the micro-environment of DC activation contributes significantly to the

outcome of T-cell polarization (165). It has been reported that the presence of IL-10 and/or the lack of IL-12 can result in a type 2 skewed response in both pDCs and myeloid DCs (166). However, until the enigma of DC plasticity versus definable subtypes is solved, it is difficult to determine the significance of microenvironment over DC type in the resulting response.

DCs are implicated in B-cell activation, although their involvement is not fully understood. DCs affect B-cells indirectly through activation of T-cells, which then provide CD40L and secreted factors to B cells. They can also capture and present unprocessed antigen to B-cells, facilitate differentiation of activated naïve B-cells into plasma cells, and enhance IgG secretion by memory B-cells by secretion of IL6Rα. BAFF/Blys, a TNF family member, is implicated in DC control of B-cell proliferation and differentiation, but the mechanism is not well understood (153).

DC maturation can be caused by NK, NKT cells, and γδT cells by a combination of cytokine and cell-cell interactions (167). In turn, DCs can activate and enhance NK, NKT, and γδT-cell function through presentation of non-peptide antigens on CD1 molecules and secretion of IL-12, IL-15, IL-2, and IFNα/β. In response, these innate lymphocytes secrete IFN-γ, which augments adaptive responses to DCs. These cells can also mature DCs in the context of a response to self antigens resulting from transformation or infection. This allows DC maturation to self peptides that would not signal through TLRs, an important mechanism in the immune response to tumors.

In addition to their immunostimulatory role DCs contribute to peripheral tolerance in the steady state by presenting self peptides without costimulation or by antigen presentation in a modified maturation state leading to the induction of T-cell anergy

and T regulatory cells (168, 169). The balance between induction of Th1 and Th2 T-cell responses also serves an immunoregulatory role. The ability of DCs to produce IL-12 is thought to be short lived resulting in a skewing towards type 2 later in the immune response (170). The antagonistic nature of type 2 cytokines to the type 1 response could serve to dampen the immune response and prevent immune inflicted pathology.

The lineage of particular subsets of DCs is not completely understood. Because tissues and methods of isolation vary, it is difficult to ascertain whether these are truly distinct populations or reflect different maturation states. Transfer of myeloid or lymphoid progenitors to irradiated hosts has been demonstrated to result in both myeloid and pDC populations, but others contend that pDCs are solely of lymphoid origin (154, 171). It has also been suggested that pDCs can differentiate into myeloid DCs (172). The teasing out of the intricacies of DC sub-classification and their evolutionary relationship to other immune cells is still very much a work in progress. The "transdifferentiation" of neutrophils into DCs has been reported by several groups (173-175), the de-differentiation of DCs into macrophages that is then irreversible has been described (176), and still others report B220+, CD11c-cells from the B-cell lineage with DC morphology and function (177). Two new reports that T-cell precursors retain the ability to become granulocytes, DCs, macrophages, or NK after they have lost the ability to become B-cells invalidates the lymphoid versus myeloid dogma, and sheds new light on the relationship between leukocytes (178, 179). This new understanding suggests that perhaps more emphasis should be placed on cell experience than predisposition. In any case, it is evident that DCs are plastic in their phenotype and function, but the extent of this plasticity remains to be seen. One author even suggests that "DCs", a population with specialized adaptations for antigen

presentation, simply do not exist. Rather than a distinct cell type, these cells merely represent mononuclear phagocytes (macrophages), that, as a result of their experience, express the machinery to present antigen to T-cells (180).

Even though there are still gaps in our understanding of DC biology, it is clear that these cells play a central role in initiating and regulating innate and adaptive immune responses. Because of this central role, a greater understanding of DCs is essential to understanding immunology as a whole. The identification of DCs or mononuclear phagocytes that perform similar functions in fish will give insight into the origins of DCs, leading to a greater understanding of DC biology and consequently immunology.

6. Dendritic Cells in Fish

With the advent of adaptive immunity, the new challenge of coordinating related but spatially and temporally distinct responses arises. In mammals, this challenge is met largely by the activities of one cell type, DCs. DCs have specialized capabilities that allow them to home to sites of inflammation, to gather information and antigen, and then migrate to secondary lymphoid tissue to present antigen to T-cells, bridging the spatial gap from sites of infection to T-cell rich areas. Once there, DCs shape the more delayed adaptive response through presentation of antigen in a cytokine and costimulatory context dictated by their experience in the periphery. In addition to stimulation, DCs can act to dampen immune responses, helping to prevent immunopathology caused by overly zealous adaptive responses.

The origin of DCs is unknown. The question as to whether DCs or a DC ancestor serving a similar function are present in fish has yet to be answered. While the similarities of mammalian and piscine immune responses form a strong argument for

the existence of a similar cell type, the differences could signal that initiation of adaptive immune responses in fish is significantly different. The demonstration of a specialized antigen presenting cell would begin to address this question. If such a cell type exists, its identification would then allow exploration of the how, when, and where of B- and T-cell responses.

There is evidence of DCs in fish, largely morphologic in nature. Long term spleen cultures yield non-adherent cells in trout that have a morphology very similar to mammalian DCs (181). These cells are poorly phagocytic and display veils and processes that are actively motile. Further functional studies were not done. A network of MHC class II-positive (and Ig-negative) cells with dendritic morphology found in T-cell rich areas of the adult nurse shark spleen are described as DCs (182). More recently, cells containing Birbeck-like granules have been described in salmonid gill, spleen, and kidney (183, 184). The restriction of Birbeck granules to a subset of mammalian DCs (Langerhans cells) suggests an evolutionary relationship between these cells. The gene homologs for CD83, a mammalian surface DC marker, have been identified in rainbow trout and nurse shark (185). Recent data suggest CD83 is more widely expressed in mammals than previously thought and plays a role in lymphocyte activation, as well as in regulation of surface MHC class II (162, 164). In addition to expression in macrophages, as previously mentioned, CD83 expression has been reported in a melanomacrophage-like cell line (81), and more recently in an Atlantic salmon non-macrophage phagocytic cell line (78) and endothelial cells (186). The widespread expression of CD83 transcripts suggests that transcript levels may not be a good marker for DCs in fish; however, CD83 transcript expression is not indicative of surface expression in mammals, so surface expression may yet be useful in identifying dendritic cells in fish once a suitable antibody has been developed (187).

The relatively recent discovery of DCs is likely a result of their rarity *in vivo* and their behind-the-scenes orchestration of the immune response. DCs were first isolated from mouse spleen in small numbers (maximum of 1.6% of total nucleated spleen cells) in the early 1970's (188). Subsequently, methods were devised of culturing larger numbers of cells *in vitro* from bone marrow (189) and peripheral blood monocytes (190), enabling extensive characterization of DCs. More recently, serum-free culturing methods have been developed for the use of *in vitro* generated DCs in vaccines to avoid generating responses to FBS (191).

7. Brief Outline of Dissertation Research

The goal of my dissertation research was to identify antigen presenting cells in the teleost model organism *Oncorhychus mykiss* (rainbow trout). I hypothesized that fish possess a cell type homologous in function to mammalian DCs. Since the primary interest was function of prospective APCs in trout, I required significant numbers of cells so that functional studies could be carried out. Mammalian DCs are a rare cell type *in vivo*, and as a result methods have been developed in mammals to culture large numbers of cells for *in vitro* manipulation. Therefore, my approach was to adapt these methods to rainbow trout in order to generate presumptive DCs for functional studies. Rainbow trout were selected based on the sole report of DCs in fish (at the time this project was conceived), which described a rainbow trout spleen culture that yielded DCs (181). The significant amount of immunology research done in rainbow trout and the availability of a gene index solidified this decision.

In Chapter 2, I describe the adaptation and optimization of mammalian DC protocols to rainbow trout. The hematopoietic cultures I developed produce a population of non-

adherent cells that resembled DCs in morphology. I went on to characterize these cells morphologically and functionally as reported in Chapter 3. I based the characterization of these cells on criteria for DCs in mammals: morphology, marker expression, response to TLR-ligands, phagocytic capacity, ability to stimulate the MLR, and migration *in vivo*. Chapter 4 summarizes these findings, discusses their relevance, and suggests further experiments to be done on these cells.

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CHAPTER 2

Development of Methods to Culture Presumptive Rainbow Trout DCs

1. Peripheral Blood Mononuclear Cultures

The identification of granulocyte/macrophage colony stimulating factor (GM-CSF) as a key growth factor for the generation of DCs allowed cultivation of these cells *in vitro* (1). The first report of generating DCs from mouse peripheral blood described an involved method of serial subculture of proliferating clumps of cells in the presence of GM-CSF. In order to study DCs in humans, a method was needed that used tissues that could be easily collected from live patients. As a result, the protocol for generation of peripheral blood monocyte derived DCs (PBMDC) was developed (2). This method used monocytes isolated from peripheral blood as progenitors. Generation of DCs was most efficient with the addition of both IL-4 and GM-CSF to the cultures. Using this method, after approximately seven days of culture, free floating clumps or individual cells composed 50-80% of cells. These non-adherent cells displayed typical DC morphology and function.

I first adapted the PBMDC protocol as it has the advantage that blood can be drawn without euthanizing the animal, allowing further studies on the same animal, and potentially, isolation of other cell types over a period of time for autologous *in vitro* experiments. Considerable experimentation resulted in the following protocol: blood was drawn into heparin from the caudal vein of multiple rainbow trout and pooled. The pooled whole blood was diluted four fold in PBS/heparin and allowed to settle for at least 20 minutes at room temperature. Leukocyte rich plasma was removed from the settled red blood cells (RBCs) and layered over Nycoprep according to the manufacturer's directions. After spinning, the buffy coat was removed and plated in $25cm^2$ flasks with phenolic caps at a density of approximately $5x10^6$ cells/ml of supplemented L-15 medium (20% FBS, 50μg/mL gentamicin, and 0.25 μg/mL fungizone). Cells were allowed to adhere for two hours, at which point non-adherent

cells were washed off and new medium was added containing mammalian IL-4 (250U/ml, human: Prospec, Israel; murine: Peprotech, Rocky Hill, NJ) and GM-CSF (1000U/ml, human: Prospec,; murine: Peprotech). Cells were "fed" additional cytokines on days two, four, and six. Non-adherent cells removed at the two hour time point were transferred to a new flask for culture without addition of exogenous cytokines. These mixed cultures (sans cytokines) yielded cells with the same morphology as the adherent culture with cytokines. A more direct comparison of adherent cultures with or without exogenous cytokines was made. No discernable differences were seen (except perhaps a slight difference in the kinetics of the cultures), and addition of cytokines was subsequently abandoned. Cells were cultured at room temperature in the atmosphere.

After removal of non-adherent cells, the cultures consisted of flattened, elongated cells adhering directly to the flask and more rounded cells that appeared attached to these cells (Fig. 2.1A). After two to three days cultures formed what appeared to be hematopoietic foci, which produced round non-adherent cells (Fig. 2.1B). Larger round monocyte-like cells took on a spiky appearance approximately 7-8 days after initiation of culture (Fig. 2.1C). Cytospins of these cultures showed a subset of cells with a strikingly similar morphology to mammalian DCs (Fig. 2.1D). Eliminating the adherence and removal step altogether did not have an adverse effect on culture yields, although it resulted in a more mixed population as a result of starting with a more mixed population of cells. It is possible that these cultures are longer lived as a result of factors produced by cells other than monocytes present in the whole culture, but this has not been experimentally addressed. Non-adherent cells from mature cultures were submitted to flow cytometry analysis, which by forward/side scatter characteristics showed two distinct populations (Fig. 2.1E). One with a low forward and side scatter

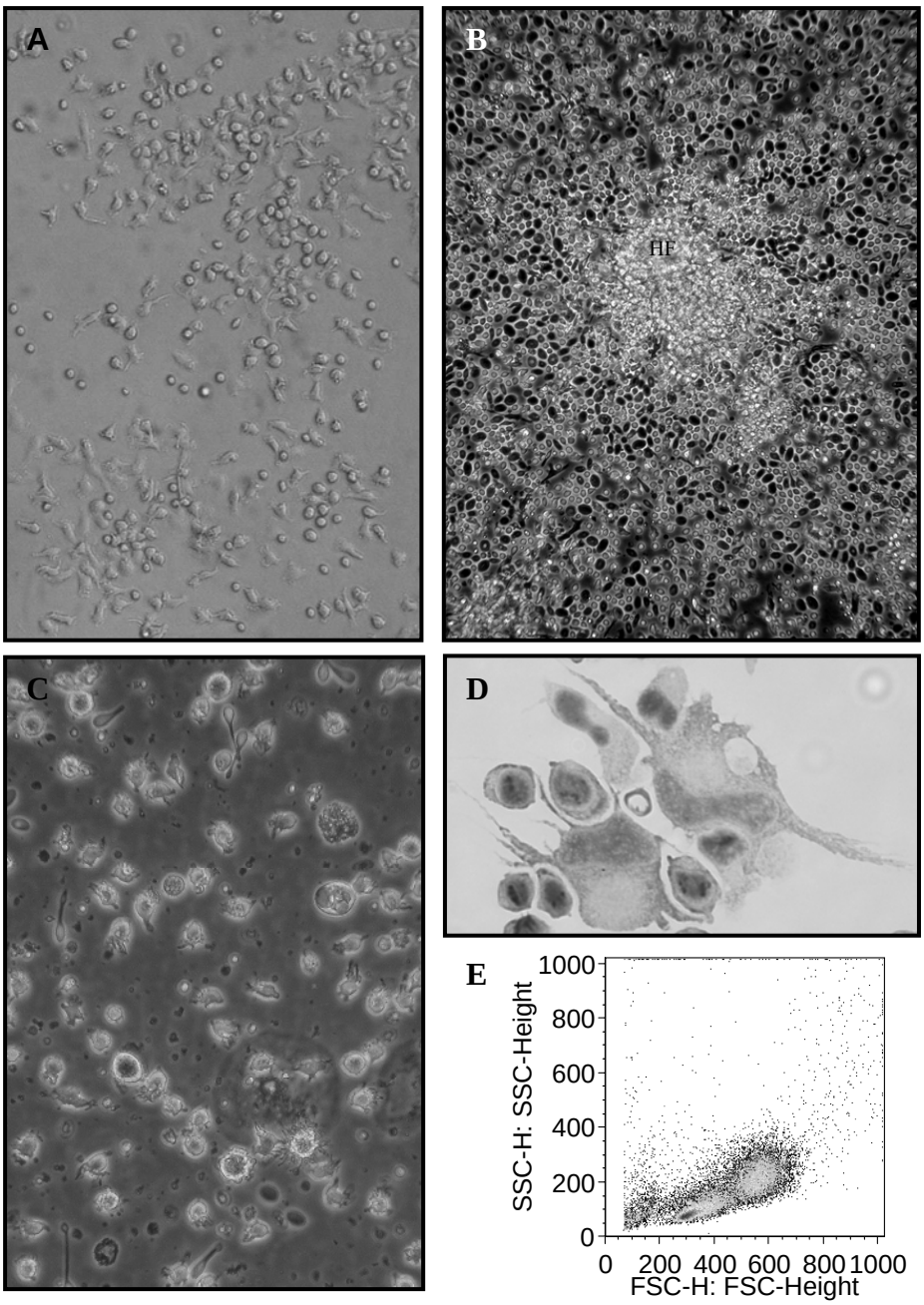

FIGURE 2.1. Peripheral blood derived DCLC cultures. (A) Adherent cells from peripheral blood monocyte fraction after 2 hour incubation. (B) Day 3 culture showing what appears to be a hematopoietic focus (HF). (C) Day 18 culture showing non-adherent cells with dendritic morphology. (D) Cytospin of non-adherent cells from day 7 of culture. (E) Forward/side scatter profile of peripheral blood culture cells.

profile typical of lymphocytes, which contained lymphocytes and the small round precursor cells found in the cultures. The other population presumably correlates to the cells with DC morphology.

While this method of culture produced cells with a morphology that resembled mammalian DCs, we were unable to generate sufficient numbers from individual fish for autologous *in vitro* experiments. The acquisition of significantly larger trout should allow cultures to be generated from individual fish, which would allow further characterization of these cells.

2. Hematopoietic Cultures

In mice, bone marrow derived DC (BMDC) cultures yield large numbers of mature DCs. The BMDC culture method was based on the hypothesis that bone marrow precursor cells with pluripotent potential could be cultured in the presence of GM-CSF in order to force the differentiation process towards generation of DCs (3). Using this method, it was reported that greater than $5x10^6$ cells could be cultured from hind limb bone marrow of one mouse. In order to adapt the BMDC protocol to trout, we used the hematopoietic tissues of rainbow trout, head kidney and spleen.

The spleen and head kidney were sterilely excised from euthanized trout and a single cell suspension was made by forcing the tissue through a 70μm cell strainer. Cells were then counted and adjusted to $6x10^6$ cells/ml in supplemented L-15 (10% BGS plus gentamicin and fungizone as above) and plated in $25cm^2$ flasks with phenolic caps. Cultures were kept at room temperature exposed to the atmosphere. Initially mammalian GM-CSF was added, but like the peripheral blood cultures, cytokine addition did not have a noticeable effect on the quality or quantity of cultures and was

not continued. Adherent cells appeared to be largely macrophages and melanomacrophages, with many round non-adherent cells also present in the culture. These round, non-adherent cells were comprised of small lymphocyte-sized cells, as well as larger monocyte/precursor-like cells. It appeared that the larger round non-adherent cells acquired dendritic morphology after approximately 1 week in culture. Like the peripheral blood cultures, what appeared to be hematopoietic foci formed in the cultures that seemed to generate additional round monocyte-like cells that then also gained a dendritic morphology over time (Fig. 2.2A). In cultures where non-adherent cells were removed, cultivation of both populations (non-adherent and adherent) gave rise to cells of dendritic morphology suggesting that they arise from adherent precursors as well as through transition from round monocyte-like cells. Cultures varied with regard to the number of such cells that developed, the time at which they emerged (typically 7-14 days), and the length of time they continued to be produced, although in some instances DCLCs were still being generated up to four months into culture. In these longer lived cultures, an adherent matrix of cells formed, but never reached confluence (Fig. 2.2B).

DCLCs can be generated, using the same protocol here described, solely from: head kidney, anterior portion of the trunk kidney, posterior portion of trunk kidney, or spleen tissue. The cultures from or containing the posterior portion of the trunk kidney contained a larger proportion of melanomacrophages and debris than other tissues. Therefore, anterior trunk kidney was not used; however, addition of the anterior portion of the trunk kidney contributed a significant number of cells without increases in melanomacrophages or debris. Large numbers of cells could be generated in hematopoietic cultures allowing characterization of these cells (see Chapter 3).

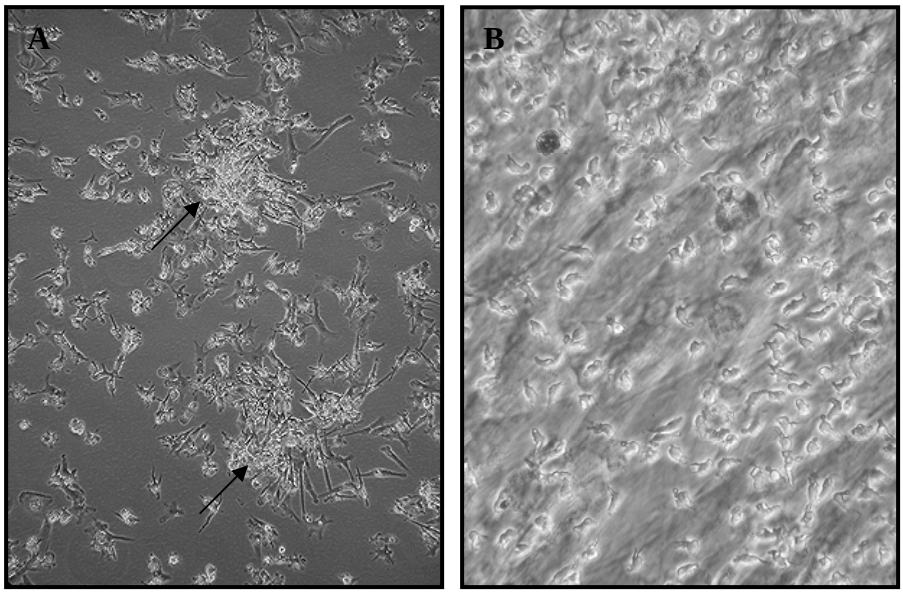

FIGURE 2.2 Hematopoietic DCLC cultures. (A) Hematopoietic foci (arrows) in cultures from head kidney and spleen tissue (non-adherent cells removed). (B) Four month old culture showing non-adherent DCLCs and a dense matrix of adherent cells.

3. Isolation of Cells From Spleen

A method for isolation of DCs from the spleen of mice was described long before the methods utilizing exogenous growth factors were developed (4). The low buoyant density fraction of cells from spleen was allowed to adhere to glass and non-adherent cells were removed. DCs lose their adherence properties when cultured, therefore, after overnight incubation of the adherent cells (some of which are adherent immature DCs), DCs became non-adherent and float in the medium. Since this method did not require the addition of cytokines, the protocol could be performed without modification. I hypothesized that if cells could be isolated in this manner, they would be equivalent to mammalian DCs.

The protocol was carried out essentially as described for mammals. Spleens were removed sterilely from euthanized trout and injected with collagenase A (100U/ml) to facilitate dissociation and recovery of adherent cells. Single cell suspensions were made, layered over Nycoprep, and spun. The buffy coat was then removed and washed

in media. Cells were counted, re-suspended, and plated in 6 well plates at a concentration of $1x10^6$ cells/ml in supplemented L-15 media (10% BGS, gentamicin, and fungizone). Cells were allowed to adhere for two hours, non-adherent cells were washed off, and new medium was added. The cultures were incubated overnight, during which time the DCs lose their adherence and come up off the plates. This can be sped up by addition of LPS or CpG in mammals. After overnight culture, the majority of the adherent trout spleen cells became non-adherent and formed free floating clumps of spiky cells, with some single floating dendritic-looking cells (Fig. 2.3A). In contrast, adherent macrophages isolated from the peritoneal cavity remained adherent after overnight incubation (Fig. 2.3B). Flow cytometry analysis demonstrated that spleen DCLC cultures comprised two populations similar to peripheral blood cultures. Cells isolated from the spleen expressed MHC class II, suggesting APC function and similarity to DCLCs from hematopoietic cultures (Fig. 2.3C).

Using this method in trout resulted in the isolation of cells bearing the same morphology as cells obtained from peripheral blood and hematopoietic cultures. The number of cells generated was very low (as is seen in mammals), and, like the peripheral blood protocol, required the pooling of spleens from several individuals, except in the case of very large trout (at least 2kg).

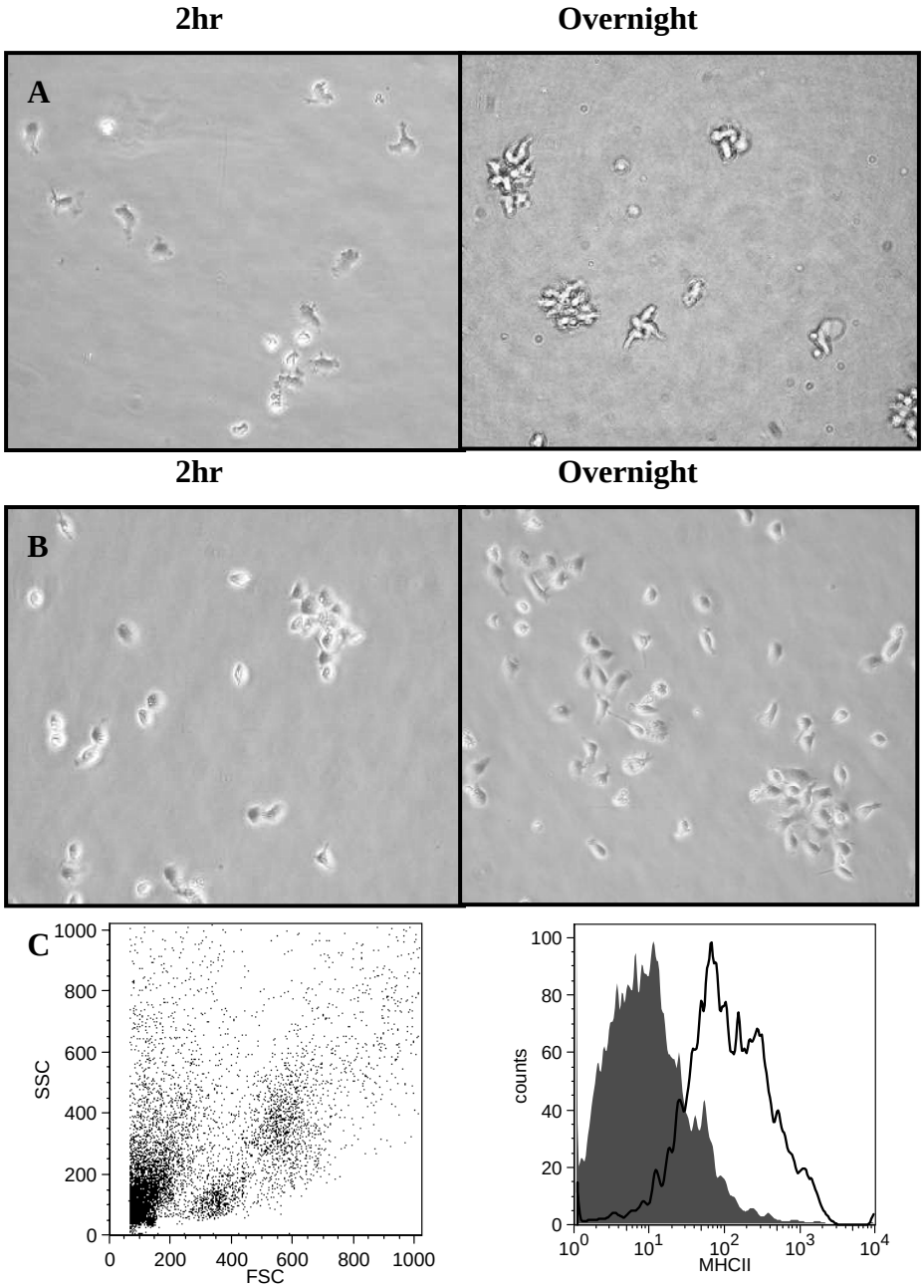

FIGURE 2.3 DCLCs isolated from spleen. (A) Adherent cells isolated from spleen at two hours and after overnight incubation. (B) Macrophages from peritoneal cavity at two hours and after overnight incubation. (C) FSC/SSC and MHC class II expression. Filled histogram was stained with pre-immune serum, while the black line is cells stained with rabbit hyperimmune serum (anti-trout MHCII).

4. Discussion

I report the adaptation of mammalian protocols for generation of DCs to rainbow trout. While PBMDC and BMDC cultures in mammals require the addition of cytokines, I was able to establish primary cultures that produced cells with dendritic morphology without addition of exogenous cytokines. It is likely that cells in the cultures produce the cytokines and growth factors necessary for DCLC production. While the culture methods I describe are similar to protocols used to generate macrophages in fish, they differ in that non-adherent cells are harvested from the cultures, rather than selecting for adherent cells. It seems likely the cells I describe may occur in macrophage cultures characterized by others, as both DCs and macrophages are monocyte-derived; however, they are consistently removed and only the adherent cells used. Therefore, the cells described here have not yet been characterized. Despite similar morphology, cells from different sources could represent distinct cell types. With the use of larger, adult rainbow trout, generation of adequate numbers of cells from peripheral blood and spleen should be possible, making comparative studies to determine if these cells are functional equivalents feasible.

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CHAPTER 3

Rainbow Trout Hematopoietic Cultures Yield Cells That Functionally Resemble Dendritic Cells*

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Abstract

Dendritic cells are specialized antigen presenting cells that bridge innate and adaptive immunity in mammals. This link between the ancient innate immune system and the more evolutionarily recent adaptive immune system is of particular interest in fish, the oldest vertebrates to have both innate and adaptive immunity. It is still unknown whether DCs co-evolved with the adaptive response, or if the connection between innate and adaptive immunity relied on a fundamentally different cell type(s) early in evolution. We approached this question using the teleost model organism, *Oncorhynchus mykiss*, with the idea that prospective DCs could be identified and characterized using a variety of functional assays. Adapting mammalian protocols for the generation of DCs, we established a method of culturing highly motile, non-adherent cells from trout hematopoietic tissue that have irregular membrane processes, much like mammalian DCs. These DC-like cells (DCLCs) express the mammalian DC markers CD83 and MHC class II, and appear capable of being activated by TLR-ligands. More importantly, trout DCLCs can stimulate the primary MLR, and when compared to other professional antigen presenting cells (APCs) elicit more efficient responses. Finally, when injected intraperitoneally into recipient trout, the cells migrate to mucosal sites. The superior antigen presenting capability seen in the MLR provides convincing evidence that these cells are specialized for antigen presentation, and support the hypothesis that they may be evolutionary precursors of the dendritic cells of mammals.

Introduction

While dendritic cells (DCs) are named for their distinctive morphology, their efficacy in stimulating naïve T-cell responses distinguishes them from the ranks of other antigen presenting cells (APCs). Their superior ability to stimulate T-cell division is driven by contact with pathogen- or death-associated molecular patterns (PAMPs, or DAMPs), typically through Toll-like receptors (TLRs). Activation involves uptake of antigen, production of cytokines, and migration to secondary lymphoid tissue. En route, DCs upregulate MHC (complexed with processed peptides), CD83, and costimulatory molecules, and at the same time lose their phagocytic capacity. Besides the physical transport, processing, and presentation of antigen, these cells collect additional information from the sites of infection through a number of mechanisms that inform their control of the adaptive response in terms of type, duration, and intensity.

Although mammalian DCs have undergone intense scrutiny in recent years, questions regarding how and when these cells evolved remain open. Jawed fish are the earliest vertebrates capable of adaptive immunity (involving BCR and TCR), and the molecular machinery necessary for antigen processing and presentation is functional in these species (1-4). There is significant evidence that activation of adaptive immune responses occurs in much the same way in jawed fish as in mammals (5). Moreover, it would stand to reason that a specialized cell type that connects innate and adaptive immunity through antigen presentation is present in lower vertebrates. Unfortunately, little is known about antigen-presentation in cartilaginous and bony fish. Indeed, such basic questions as where antigen-presentation takes place and which cells are primarily responsible for stimulating T-cell proliferation are still unanswered.

Despite this, several observations provide tantalizing evidence that cells homologous to the dendritic cells of mammals may exist in fish. These include the description in nurse shark of a network of MHC class II positive cells in the T-cell rich areas of the spleen (6); a long-term trout splenic culture that yields non-adherent cells designated as DCs based on their morphology (7); the identification of Birbeck-like granules in cells of the gill epithelium and lymphoid tissue of salmonids (8); and more recently, the description of a dendritic-like phagocytic cell line from Atlantic salmon (9). While these papers make a case for the existence of DCs, or a DC homolog in fish, the claim is tenuous without further functional characterization, specifically of their ability to present antigen. Therefore, we sought to identify and functionally characterize prospective DCs to shed light on the question of which cell types are principally involved in the mounting of an adaptive response in fish. In this paper, we describe a homogenous population of cells cultured from rainbow trout hematopoietic tissue that bear the essential hallmark of mammalian DCs: a superior ability to stimulate the MLR compared with other established APCs. Our data suggest the presence of a specialized APC in trout that may be the elusive link between innate and adaptive immunity in lower vertebrates.

Materials and Methods

Fish maintenance

Rainbow trout were obtained from Tunison Laboratory of Aquatic Science, Cortland, NY; New York State Department of Environmental Conservation Bath fish hatchery, Bath, NY; Carpenter's Brook Fish Hatchery, Elbridge, NY; and Fish Haven Farms, Candor, NY. All trout were maintained in flow-through tanks at an average temperature of 13˚C. Fish were fed commercial trout chow once/day.

Cell culture

200-2000g rainbow trout were euthanized by overdose on MS-222 (Finquel, Redmond, WA). Fish were bled from the caudal vein prior to sterile removal of the spleen, head kidney, and anterior portion of trunk kidney; defined as segment between head kidney and Corpuscles of Stannius. Including regions posterior to the Corpuscles of Stannius resulted in excessive numbers of melanomacrophages in the cultures. Excised tissue was placed in L-15 media (Invitrogen, Carlsbad, CA) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT), gentamicin (50µg/ml, Invitrogen, Carlsbad, CA), and fungizone (0.25 µg/ml, Invitrogen) (supplemented media) on ice. Tissue was broken up with the stopper of a 1ml syringe. Disrupted tissue was then forced through a 70µm cell strainer (BD, Franklin Lakes, NJ) resulting in a single cell suspension. Cells were counted and adjusted to a final concentration of approximately $6x10^6$ live cells/ml (as determined by trypan blue exclusion) in supplemented media and plated in $25cm^2$ or $75cm^2$ cell culture flasks with phenolic style caps (Corning, Corning, NY). Cells were cultured at room temperature for 7-30 days before harvest. In some cases, cells could be harvested several times in that period replacing the old medium with fresh supplemented media.

Harvest and Enrichment of DCLCs

Cell culture flasks were agitated to suspend non-adherent cells and the media was collected and pooled. The cell suspension was layered over Nycoprep/One-step monocytes (Accurate chemical, Westbury, NY) and spun as per manufacturer's instructions. The buffy coat, which was enriched for DCLCs, was removed and washed twice in media prior to use.

Serum collection

Euthanized trout were sterilely bled from the caudal vein and the blood was allowed to clot at room temperature for 1-1.5 hours and then placed on ice. Blood was spun at 2000xg for 10min at 4˚C and serum collected with a serological pipette prior to storage at -80˚C. Serum samples were later thawed, pooled, diluted to 20% in L-15 media, and filter sterilized. The diluted serum was then heat inactivated at 56˚C for 20 min, aliquoted, and returned to -80˚C until use.

Phagocytosis Assay

Green fluorescent latex beads (1µm; Sigma, St. Louis, MO) were opsonized by adding 5µl beads per 100 µl L-15 medium supplemented with 10% trout serum and incubating for 1 hr at RT. Cells were suspended in supplemented media at a concentration of $1x10^7$ cells/ml and plated in 96 well flat bottom tissue culture plates (Corning, Corning, NY). An equal volume (100µl) of opsonized beads was added to cells and plates were spun at 600xg for 2 minutes to bring the beads into contact with the cells. Following incubation for 2hrs at RT, cells were washed 3x with PBS/0.1%BSA and re-suspended in 500µl ice cold PBS/BSA until flow cytometry analysis. Initially, 0.4% trypan Blue was added to tubes to quench the fluorescence of beads that had not been internalized, however, flow data collected from samples before and after addition of trypan blue were indistinguishable and the trypan blue addition was subsequently abandoned. Cytospins also showed beads that had not been internalized were successfully removed in the wash steps.

Flow cytometry

Cells were maintained at 4˚C throughout. Cells were blocked in 10% goat serum (Zymed, South San Francisco, CA) for 15 min, stained in primary antibody for 20min,

and in secondary antibody for 20min. Cells were then washed 2x and re-suspended in 400μL of ice cold PBS/0.5%BSA and placed on ice until flow cytometry was conducted. Data collection was performed on a FACSCalibur (BD, Franklin Lakes, NJ) using Cellquest software (BD). FloJo software was used for data analysis (Treestar, Ashland, OR). Antibodies were diluted in PBS/0.5% BSA/0.1% sodium azide. Primary antibodies used were: monoclonal antibody 1.14 (10) against rainbow trout IgM at 1:500 (kind gift from Dr. Stephen Kaattari, Virginia Institute of Marine Sciences); anti-trout MHCII β hyperimmune rabbit serum at 1:4000; and anti-thrombocyte antibody S5H2 (11) at varying dilutions; neat, 1:10, 1:100, 1:1000, and anti-thrombocyte antibody 28D7 (11) at 1:200 (both kind gifts from Dr. John Hansen, University of Washington/USGS-Western Fisheries Research Center, Seattle,WA). Secondary antibodies used were: goat anti-rabbit FITC (Invitrogen, Carlsbad, CA) at 1:200; goat anti-mouse FITC (Invitrogen) at 1:200; and goat anti-mouse AlexaFluor-633 (Invitrogen) at 1:500.

Microscopy and TEM

Phase contrast images of cultures were taken on an Olympus CK2 inverted microscope with an Olympus SP-350 digital camera (Olympus, Center Valley, PA). All other micrographs were taken using a Zeiss Axioimager M1 microscope using Axiovision 4.6 software, and an Axiocam HR camera (Carl Zeiss, Germany).

TEM was performed by the Cornell Integrated Microscopy Center. Double-strength glutaraldehyde (4%) was added to an equal amount of DCLC suspension. After the primary fixation, the cells in the suspension were pelleted, the glutaraldehyde removed, and buffer (0.1 M sodium cacodylate solution, 7.4 pH) was added. The cells were then re-suspended by gentle agitation. DCLCs were rinsed three to six times for 10 minutes each. Material was then placed in a 1% osmium tetroxide solution (diluted

to 1% with the sodium cacodylate buffer) for an hour at room temperature. Material was rinsed three to six times in a 0.1 M sodium cacodylate buffer wash for 10 minutes each. Next the material was dehydrated starting with a 10% ethanol solution, and then moved through 30%, 50%, 70%, and 90% solutions. The material was in each of these for at least 10 minutes in a cold environment. Following dehydration the material was rinsed twice in 100% ethanol, for 10 minutes each at room temperature. The material was then rinsed in a 1:1 ethanol and acetone solution for 10 minutes followed by two rinses in 100% acetone for 10 minutes each. Next the material is infiltrated with plastic using solutions of acetone and epon-araldite, with gradually increasing concentrations of the plastic. The material was agitated constantly while being infiltrated with the plastic. First a 1:4 solution of plastic and acetone was used. The material was left in the solution for 1/2 to 1 hour. Next were a 1:2 solution (1 to 2 hours), a 1:1 solution (at least 2 hours), a 2:1 solution (2 to 3 hours), a 4:1 solution (3 to 4 hours), and finally, pure plastic overnight. Accelerant was mixed into the plastic and the material was left for 4 hours. The material was then imbedded in blocks and baked in the oven overnight at 60 degrees. After the blocks were removed from the oven, ~70nm sections were taken with a Reichert OmU2 ultramicrotome. The sections were then contrasted using uranyl acetate and lead citrate. The grids were viewed in a Tecnai 12 Biotwin microscope; the images were acquired using a Gatan Multiscan camera, model 791.

RNA preparation

RNA was extracted from tissues in either Trizol (Invitrogen, Carlsbad, CA) or RNeasy buffers (Qiagen, Valencia, CA). Trizol extractions were performed as per the manufacturer's directions except for the inclusion of an additional phenol step. After the initial phenol/chloroform extraction, an equal volume of low pH phenol (Ambion,

Austin, TX) was added to the aqueous phase, and the sample shaken vigorously for 15 seconds before centrifugation at 12000xg for 10 min at 4˚C to separate the phases. Following ethanol precipitation and dissolution in H_2O, Trizol extracted RNA was treated with RNase-free DNase (DNA-Free Turbo kit; Ambion) according to the manufacturer's protocol, re-precipitated with ethanol, and dissolved in RNAse-free water prior to storage at -80^{o}C. RNeasy extractions were performed according to the manufacturer's protocol. RNA eluted from columns was subjected to DNase treatment as described above and cleaned up using the RNeasy kit (Qiagen). In all cases, RNA was quantitated by UV-spectrophotometry and subjected to electrophoresis on agarose gels to verify integrity.

Real Time RT-PCR

Primers (Sigma Genosys, The Woodlands, TX) and probes (5' 6-FAM, 3' TAMRA labeled, Eurogentec, San Diego, CA) with the following sequences were designed with Primer Express software (Applied Biosystems): CD83 forward AGCAGAGGAACATGTCGATGC; CD83 Reverse GGCCAACCGATG-TTCAAAAT; CD83 probe AATTGCCTTCTACATTTGCCTGACCTTGATTT; MHCII forward AGCAGAGGAACATGTCGATGC; MHCII reverse GGCCAACCGATGTTCAAAAT; and MHCII probe AATTGCCTTCTACA-TTTGCCTGACCTTGATTT. Sequences used for primer and probe design were accessions: AY263797 (CD83), and U20947.1 (MHCII). Primers were used to generate PCR products from rainbow trout cDNA which were blunt cloned into ZeroBlunt TOPO (Invitrogen, Carlsbad, CA). Cloned inserts were sequenced and orientation of inserts was determined. Appropriate restriction enzymes were then used to linearize the plasmid and T7 or SP6 RNA polymerase (Promega, Madison, WI) was used to generate RNA transcripts *in vitro*. Transcripts were DNase treated to digest the

plasmid, followed by ethanol precipitation to remove digested fragments and nucleotides. RNA was quantitated and subjected gel electrophoresis to check size and integrity. The copy number was calculated and the transcripts were diluted into single use aliquots (ten-fold dilutions were made from 10^8 -10 copies per reaction volume) in yeast tRNA (6.25 ng/µl), and stored at -80°C until use. The transcripts served as quantitative standards in Real Time RT-PCR reactions. One-step Real time RT-PCR was done on an ABI 7500 Fast Real-Time PCR System (ABI, Foster City, CA) using the following conditions: 30min 48°C for RT, 10min 95°C for polymerase activation, followed by 40 cycles of 15s at 95°C, then 1 min at 60°C. Reactions were performed in triplicate (for samples and duplicate for standards) in 96-well optical plates (ABI), using 17µL master mix (one-step master mix (ABI) plus: 0.3µM forward primer, 0.3 µM reverse primer, and 0.2 µM probe) and 8 µL of: RNA sample (50ng total RNA), standard, or water in the case of no template controls. To control for genomic DNA contamination real time RT-PCR was done on each sample without the addition of reverse transcriptase. Product was not detected in the absence of template in control wells. Data analysis was done with ABI's built-in Sequence Detection System (v.1.4).

TLR-ligand treatment

Cells were stimulated for 24hrs with a mixture of ssRNA (2µg/mL), Imiquimod (10µg/mL), Flagellin (from *S.typhimurium*, 100ng/mL), and poly I:C (25µg/mL) (all from Invivogen, San Diego, CA) in supplemented L-15 medium.

Isolation of cells for MLR

IgM+ (B-cell stimulators) and IgM- (responders): Spleens were aseptically collected from euthanized trout and forced through a 70µm cell strainer to form a single cell suspension. Cell suspensions were then fractionated on Accupaque gradients

according to the manufacturer's instructions (Accurate Chemical, Westbury, NY). After centrifugation, the buffy coat containing the lymphocyte fraction was removed and pooled. Cells in this fraction were used as responders in initial DCLC MLRs. For MACS® sorting into IgM+ and IgM- fractions, this fraction was stained as for flow cytometry using mAb 1.14 (10) and FITC-labeled secondary antibody, then washed and incubated with MACS® anti-FITC beads (10μL/10^7 cells) for 15 min. Cells were re-suspended in 3mL PBS/0.5%BSA and MACS® sorted using midi-MACS columns (Miltenyi Biotec, Germany). IgM+ (B-cell) and IgM- (responder) fractions were collected, washed, and counted for use in subsequent experiments. Resident Peritoneal Macrophages: Peritoneal lavage fluid was collected, spun at 400xg for 10min and the resulting cell pellet re-suspended in 5-15ml supplemented media. Cells were plated in a 6-well plate (Corning, Corning, NY) and incubated at room temperature from 2hr to overnight depending on the experimental requirements. Non-adherent cells were washed off and the remaining adherent macrophages were collected by incubating in ice cold PBS/BSA without Ca++ or Mg++ for 10 min and harvested with a cell scraper (Corning, Corning, NY).

MLR

All procedures were carried out at 4°C except labeling, which was done at room temperature. IgM-negative responder cells and stimulator cells (B-cells, DCLCs, and macrophages) were counted and re-suspended at a concentration of $1x10^6$cells/ml in PBS. Responders were labeled with 10μM CFSE (Invitrogen, Carlsbad, CA) for 15 minutes, and stimulators with 10μM Celltrace Far Red (Invitrogen) for 20min. Five volumes of ice cold L-15 was then added to stop labeling and cells were incubated on ice for 5 min, followed by 2 washes in PBS. Cells were re-suspended at $2x10^6$cells/ml in L-15 medium containing gentamicin, and fungizone (as above) and supplemented

with 5% trout serum (MLR medium). Stimulators were then plated in 100µl MLR medium in triplicate at $2x10^5$, $2x10^4$ and $2x10^3$ cells per well in 96-well round bottomed plates (Corning). A total of $2x10^5$ responders were then added to all wells resulting in stimulator:responder ratios of 1:1, 1:10, and 1:100. Negative control wells contained $4x10^5$ responders alone. MLR plates were incubated for 6 days (determined to be optimal in a time course experiment carried out to day 8). On day 6, triplicates were pooled and re-suspended in PBS/BSA for flow cytometry. Data were collected and analyzed by excluding far red-positive cells (stimulators) in order to differentiate divided responders from CFSE-negative stimulators. Responders without stimulators were used to draw a gate for dividing cells as any division seen in these wells was considered background (less than 2.0% dividing cells was seen in all experiments).

Transfer Experiments

Enriched MCs were stained as above with CFSE for 20 min then washed 2x in L-15, followed by a final wash in PBS. A total of $2\text{-}4x10^7$ labeled cells was injected i.p. per fish in ~250µL PBS. Twenty four hours after injection, fish were euthanized and blood, skin, gill, spleen, gut, head kidney, anterior kidney, posterior kidney, liver, and swim bladder tissue as well as peritoneal lavage fluid were collected in PBS/BSA/EDTA and placed on ice. Single cell suspensions were generated by passage through a 70µm cell strainer (BD Falcon, Bedford, MA, USA). Certain tissues required additional treatment prior to passing through a cell strainer: spleen was treated with collagenase A (100 U/ml, Sigma, St. Louis, MO) at room temperature, swim bladder was washed off in PBS, cut open, and cells were scraped off the interior surface with a scalpel and placed in media on ice. The remainder was cut up and then placed in PBS/0.5%BSA/Trypsin/EDTA/collagenase A (digestion solution) (Invitrogen; Serological Proteins Inc, Kankakee, IL; 0.5%, Invitrogen, Carlsbad, CA;

and 100 U/ml, Sigma respectively). Similarly, gut was repeatedly rinsed out with PBS/BSA using a 10mL serological pipette, cut open, and the surface cells were scraped off with a scalpel and placed on ice. The remaining tissue was cut up and placed into digestion solution. Skin (cut into small pieces) and gill (whole arches) were also placed in digestion solution. Incubations in digestion solution were on a shaker at room temperature for approximately 1-1.5 hours before generation of a single cell suspension. Suspensions from each tissue were strained though a 40μm cell strainer (BD Falcon, Bedford, MA, USA) prior to flow cytometry. $1x10^6$ events (cells within live gate based on FWD-SSC scatter) were collected per tissue. The un-migrated CFSE positive cells remaining in the peritoneal cavity were used to generate a gate, which was used to determine the number of CFSE positive cells in each tissue per one million events. The number of positive cells that had migrated was totaled for each fish and the percent of that total was determined for each tissue to arrive at a % migrated cells per tissue.

Results

Hematopoietic cultures yield non-adherent cells with dendritic morphology.

Primary cultures were started from single cell suspensions of head kidney, anterior trunk kidney and spleen of rainbow trout. Within 24 hours, many of the cells had adhered to the flask and formed clumps. Adherent cells were comprised primarily of melanomacrophages and macrophages. Small, round, non-adherent lymphocyte/thrombocyte cells were also present in variable numbers and persisted throughout. Thrombocytes typically assumed a spindle-shaped morphology and formed floating clumps.

In addition to non-adherent lymphocytes and thrombocytes, larger, round, non-adherent monocyte/progenitor cells were present in the culture that increased in number over time. By 1-2 weeks, these cells had assumed a branched morphology reminiscent of the dendritic cells (DCs) of mammals (Fig. 3.1A-B).

Given their tissue of origin and gross morphology, we were intrigued by the possibility that these DC-like cells (DCLCs) could be the trout homolog of mammalian dendritic cells. As with mammalian DCs, we found that we could enrich for these low buoyant density cells by isopycnic separation on 1.068g/mL separation medium (12). Prior to separation, primary cultures contained mixtures of cells that could be readily distinguished by flow cytometry (Fig. 3.1C). After enrichment cells became far more uniform in size and granularity/complexity, with the major population having a similar forward/side scatter profile to that of monocytes/macrophages/DCs in mammals (Fig. 3.1D).

Cytospins of enriched DCLCs showed homogenous populations of cells with irregular shape and lobular nuclei (Fig. 3.1E). Transmission electron microscopy (TEM) offered a more detailed view of these cells. Multi-lobular nuclei with condensed chromatin at the margins were typical, as were long cell processes that extended many microns from the periphery. DCLCs were also rich in tubular ER and had characteristic vesicles containing electron dense material or in some cases, lamellar bodies of unknown structure and function (Fig. 3.2).

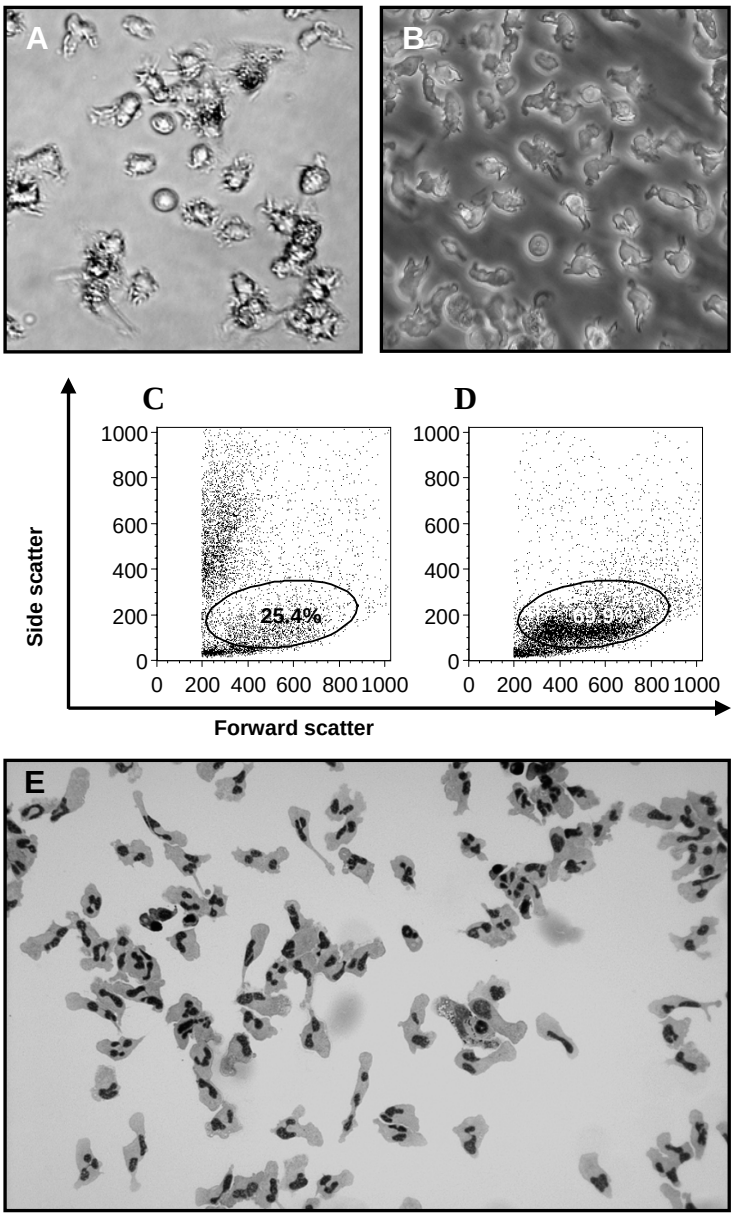

FIGURE 3.1. **Culture and enrichment of DCLCs.** Phase bright (A) and phase contrast (B) images of hematopoietic cultures showing non-adherent cells with dendritic morphology. Flow cytometry forward/side scatter profile of cultured cells before (C) and after (D) enrichment. Gates indicate % DCLC population; typical purity after enrichment was approximately 80 percent. (E) Cytospin of enriched cells showing a homogenous population of cells with multi-lobed nuclei and irregular morphology.

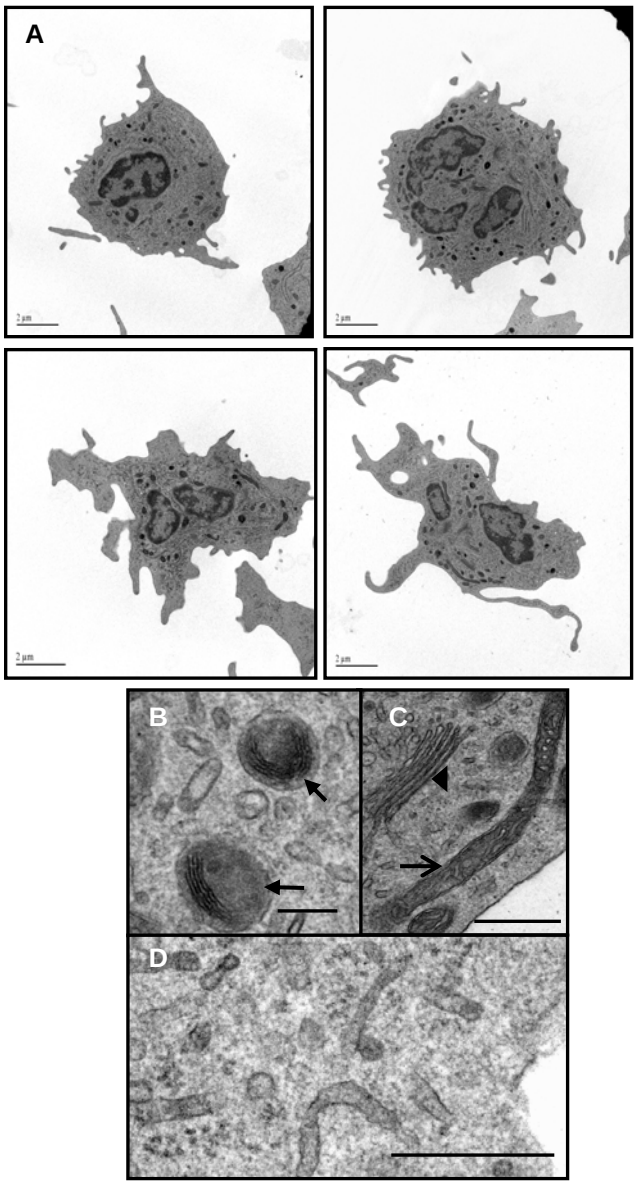

FIGURE 3.2. **TEM of DCLC.** (A) Transmission electron micrographs of representative DCLCs. Cells have multi-lobed nuclei and numerous cytoplasmic extensions and veils (scale bars=2 μm). (B) High magnification of cytoplasm detailing lamellar structures seen in some cells (arrows). (C) High magnification of tubular mitochondria (arrow), Golgi (arrow head) and smooth ER (D) characteristic of DCLCs. Scale bar in B=0.2μm, C and D=0.5μm.

Mammalian DCs are highly motile and continually "taste" their environment by extending and contracting cytoplasmic veils and processes. Consistent with this behavior, trout DCLCs showed active motility, extending and retracting processes as

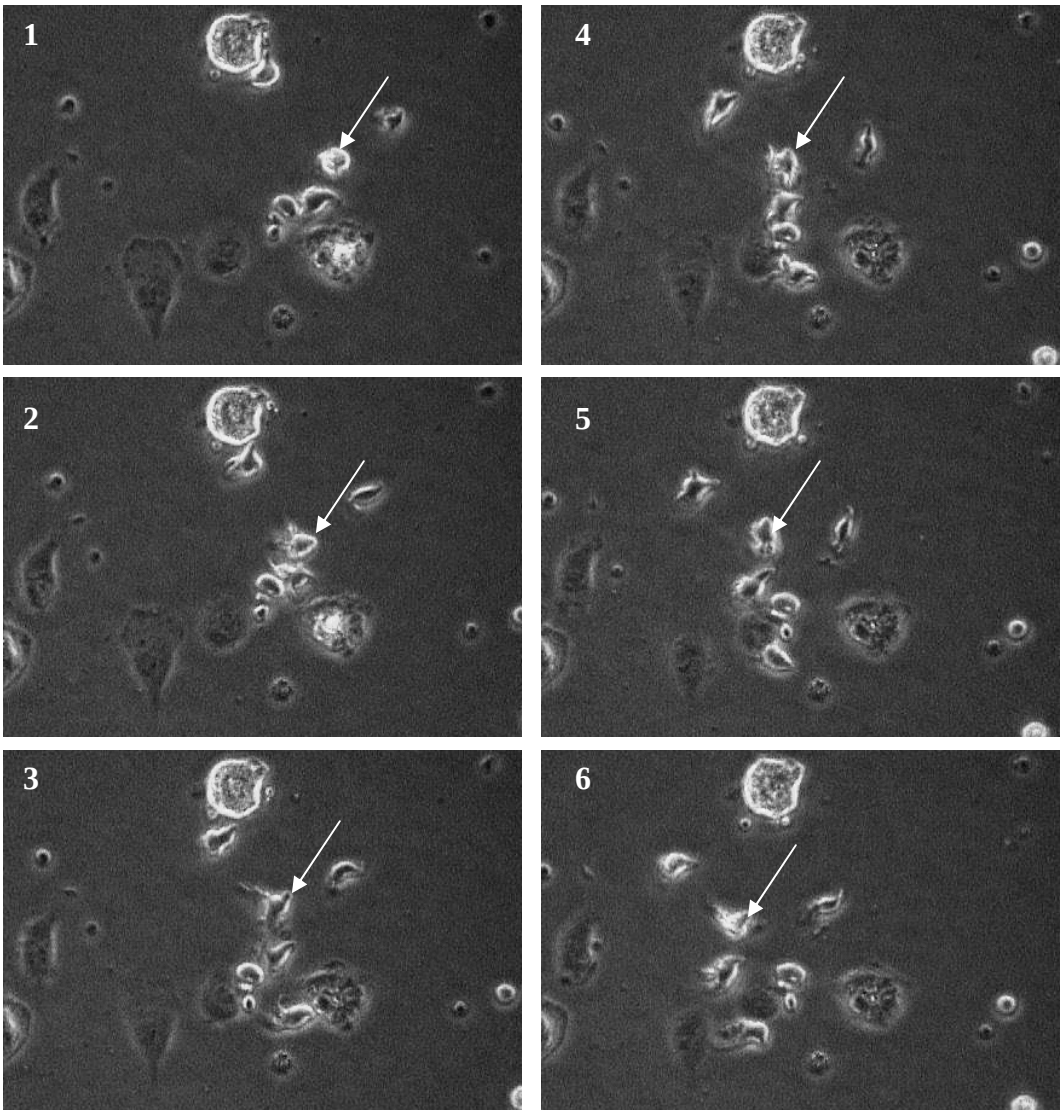

FIGURE 3.3. DCLC Motility. DCLC are motile cells moving constantly in culture. Numbers 1 through 6 indicate the sequence of still images from a movie of DCLCs in motion. The white arrow indicates the same cell in each frame. The cell extends and contracts processes and moves across the frame.

they moved (Fig. 3.3). In contrast, adherent macrophages demonstrated amoeboid-like movement that was uncharacteristic of DCLCs.

Expression of surface markers

To determine whether trout DCLCs expressed markers that would be expected for APCs, we stained cells with rabbit hyperimmune serum against rainbow trout MHC class II. As shown in Figure 3.4, DCLCs expressed uniform levels of MHC class II on their surface but failed to stain with antibodies against trout IgM. Thrombocytes have been reported to express MHC class II transcripts (11, 13) in trout, but cultured DCLCs failed to stain with previously characterized anti-thrombocyte antibodies (data not shown). Finally, gene homologs to the mammalian DC marker CD83 have been described in rainbow trout (14). When examined by real time quantitative RT-PCR, DCLCs expressed abundant levels of CD83 mRNA transcripts compared with muscle, a tissue expected to be relatively free of antigen-presenting cells (Fig. 3.4C).

Response to toll-like receptor-ligands

The unique activation program of DCs is triggered by exposure to toll-like receptor-ligands (TLR-ligands) and other PAMPs. Although TLRs have been identified in trout and other fish, it was unclear which (if any) TLRs were expressed in DCLCs. Therefore, we treated DCLCs with a mixture of four TLR-ligands (imiquimod, PolyI:C, ssRNA, and flagellin) and observed their response at the cellular level. After twenty-four hours, DCLCs showed marked aggregation (Fig. 3.5B), while cells in media alone were randomly distributed (Fig. 3.5A). This aggregation is common to activated leukocytes and has been described in mammalian and trout cells alike (15, 16). At the molecular level, significant ($p<0.05$) increases in CD83 mRNA transcripts were observed in DCLCs treated with TLR ligands compared with media alone (Fig. 3.5C). Interestingly, no increases in the expression of MHC class II transcripts were seen (Fig. 3.5D). At the twenty-four hour time point, surface MHC class II expression was unchanged following exposure to TLR-ligands (data not shown). To examine this

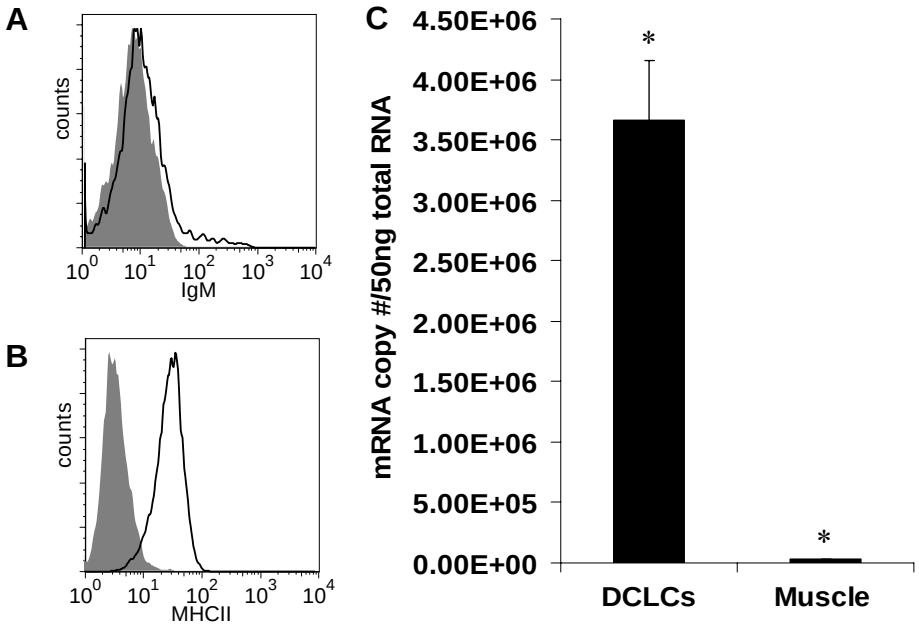

FIGURE 3.4. DCLCs express MHCII and CD83, but not IgM. (A) DCLCs do not stain for IgM. Grey histogram is cells stained with secondary antibody alone and the black line is cells stained with anti-IgM (Warr's 1-14 antibody) followed by secondary antibody. (B) DCLCS do stain positive for MHC class II, grey histogram is cells stained with pre-immune serum followed by secondary antibody, and the black line is cells stained with MHC class II hyper-immune serum and secondary antibody. Data are representative of five experiments. (C) CD83 mRNA expression in DCLCs compared to tissue that is not expected to contain APCs (muscle). (*) Indicates statistical significance ($p<0.05$). Data are representative of three experiments. Error bars represent SD of real time RT-PCR triplicates.

further, we performed time course studies and found that DCLC MHC class II surface expression increased on day 4 of TLR-ligand treatment (Fig. 3.5E).

Phagocytosis

In mammals, immature DCs are phagocytic but lose this ability upon activation with TLR-ligands. DCLCs from different fish were examined for their ability to take up 1μm fluorescent beads. After two hours of incubation with opsonized beads, a majority of DCLCs had phagocytosed beads, indicated by increased fluorescence and

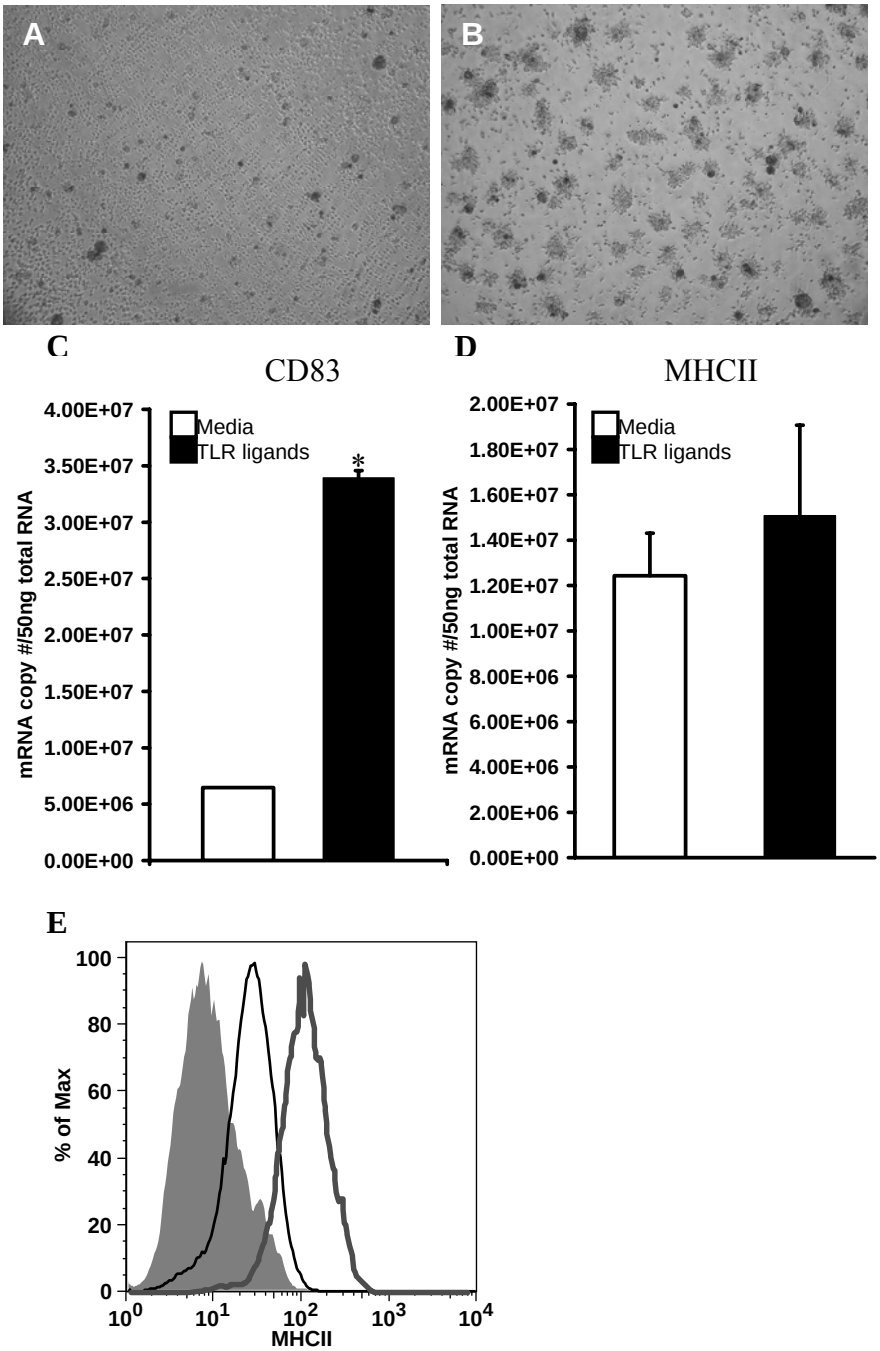

FIGURE 3.5. DCLCs respond to TLR-ligands. Phase contrast images of DCLC incubated in medium alone (A) or medium with TLR-ligands for 24hrs (B*)* (10x). Messenger RNA transcript levels of CD83 (C) and MHC class II (D) in DCLC cultured with or without TLR-ligands for 24 hrs. Data are representative of four experiments. Error bars indicate SD of RT-PCR triplicates. (E) DCLC upregulate surface expression of MHC class II four days after exposure to TLR-ligands. Grey filled histogram is cells stained with pre-immune serum and secondary antibody, the black line is DCLCs incubated in medium for four days stained with hyperimmune serum, while the thick grey line is day four of DCLC exposure to TLR-ligands.

an increase in side scatter (Fig. 3.6A,B). DCLCs were treated with TLR-ligands to determine their effect on phagocytosis. Although changes in phagocytic activity were seen, results were inconsistent; both increases and decreases in phagocytic capacity were seen with treatment (Fig. 3.6C-F).

Antigen presentation

The defining feature of DCs is their unrivaled efficacy in activating naive T-cells, which was first demonstrated in the primary MLR (17). We therefore examined whether DCLCs were capable of antigen presentation in allogeneic primary MLRs using various ratios of DCLC stimulators to spleen responder cells. Responders incubated without DCLCs had a background of 0.45% dividing cells. As the ratio of DCLCs to responders increased, the percent dividing cells increased in a dose dependent manner. At the highest ratio (1:1) DCLCs stimulated responder cells to divide with high efficiency (Fig. 3.7).

Having established that DCLCs could trigger division of splenic responders, we performed similar assays to compare these cells to other professional APCs, namely, macrophages and B-cells. Resident peritoneal macrophages were obtained by peritoneal lavage followed by adherence to plastic, and B-cells were obtained from a splenic lymphocyte fraction by MACS sorting of IgM+ cells. In this case, we used negatively selected IgM- spleen lymphocytes as responders. Since there are no markers that can be used to differentiate T-cells that have divided numerous times ($CFSE^{low}$ cells) from unstained stimulators, stimulators were stained with a persistent far-red dye and gated out in the analysis. When the ability of DCLCs, B-cells, and macrophages to stimulate the same responder cells was compared, DCLCs consistently stimulated more proliferation (Fig. 3.8).

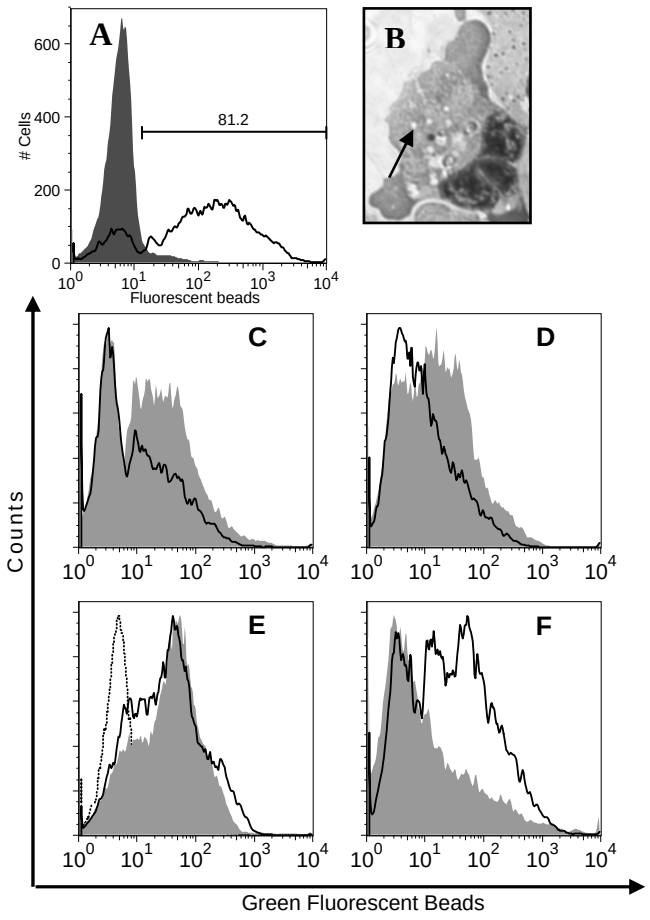

FIGURE 3.6. DCLCs phagocytose opsonized 1µm latex beads. (A) Fluorescence of DCLCs incubated for 2 hours with fluorescent beads (black line) compared to DCLCs alone (grey filled histogram). Gate indicates percent phagocytic cells. Representative data are shown (mean percent phagocytic DCLCs for experiment was 68.6%, n=5). Experiment was repeated 2 times. (B) Cytospin showing internalized beads (black arrow indicates one bead in the cell). (C-F) Phagocytosis after 24 hrs in medium (shaded in grey) or with TLR ligands (black lines, cells without beads: dotted histograms) was determined. Treatment resulted in a decrease in phagocytosis in some cases (C&D), a small shift in the most phagocytic cells (E), or an increase in phagocytic capacity (F). Data are from three experiments.

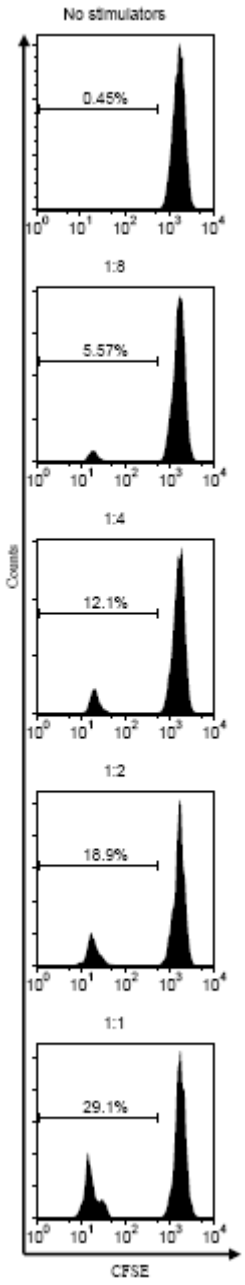

FIGURE 3.7. DCLCs stimulate the MLR. Spleen responder cells were stained with CFSE, mixed with DCLCs at varying ratios, and incubated for 6 days. DCLC to responder ratios are noted above each histogram. Gates indicate percent of responders that underwent division. Division of responders was dose dependent on the concentration of DCLCs used as stimulators.

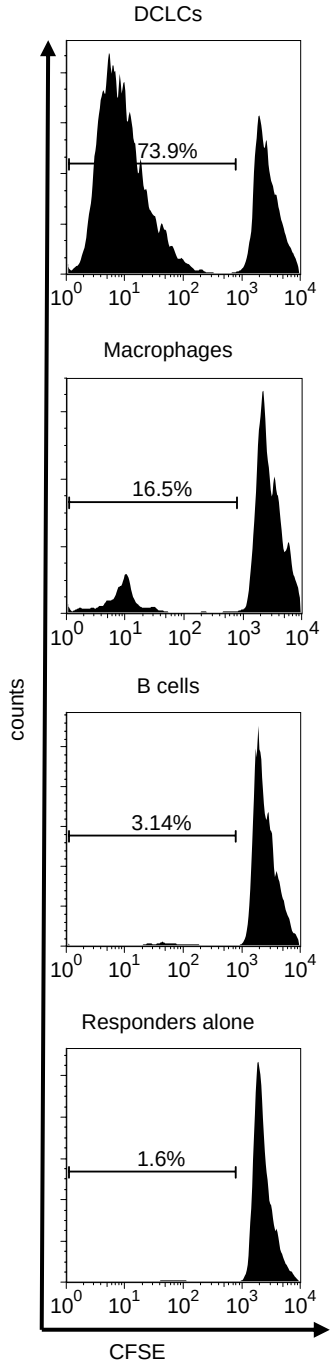

FIGURE 3.8. DCLCs stimulate the MLR more effectively than B cells or macrophages. Allogeneic primary MLRs were set up with DCLCs, macrophages, B cells, or no stimulators. Six days later CFSE stained responders were analyzed by flow cytometry for percent dividing cells. Data are representative of at least two experiments.

Migration in vivo

Mammalian DCs are located in the periphery, most notably in the skin and mucosal surfaces where they encounter pathogens. While the sites of antigen presentation in fish have yet to be characterized, the spleen and kidney are considered likely sites of T-cell–APC interactions. To determine if DCLCs migrate to putative sites of antigen presentation, we transferred CFSE stained cells to allogeneic fish to observe their migration *in vivo*. 24 hrs after intraperitoneal injection, the number of CFSE-positive cells in various tissues of recipient fish was ascertained by flow cytometry. Surprisingly, a higher percentage of CFSE-positive cells was recovered from the swim bladder than any other tissue (Fig. 3.9A). Aside from swim bladder, the gut had the highest percentage of recovered cells followed by the remaining tissues, which contained similar percentages of cells. We conclude that the cells migrated primarily to mucosal sites. To determine if activated cells would migrate preferentially to different tissues, DCLCs were treated with TLR-ligands for twenty-four hours, washed, and injected into recipients. Twenty-four hours after injection the fish were euthanized and analyzed. Interestingly, the highest percentage of cells migrated to the spleen in this case (Fig. 3.9B).

Discussion

Under the conditions described here, single-cell suspensions from head kidney, anterior portion of trunk kidney, and spleen of rainbow trout give rise to populations of non-adherent cells with low buoyant density that are highly motile in culture. As with dendritic cells in mammals, these cells extend slender pseudopods, respond to TLR-ligands, are phagocytic, and express both CD83 and MHC class II genes. Finally, when mixed with allogeneic spleen cells, they

induce potent proliferative responses compared to macrophages and B-cells, strongly suggesting that they are specialized for antigen presentation.

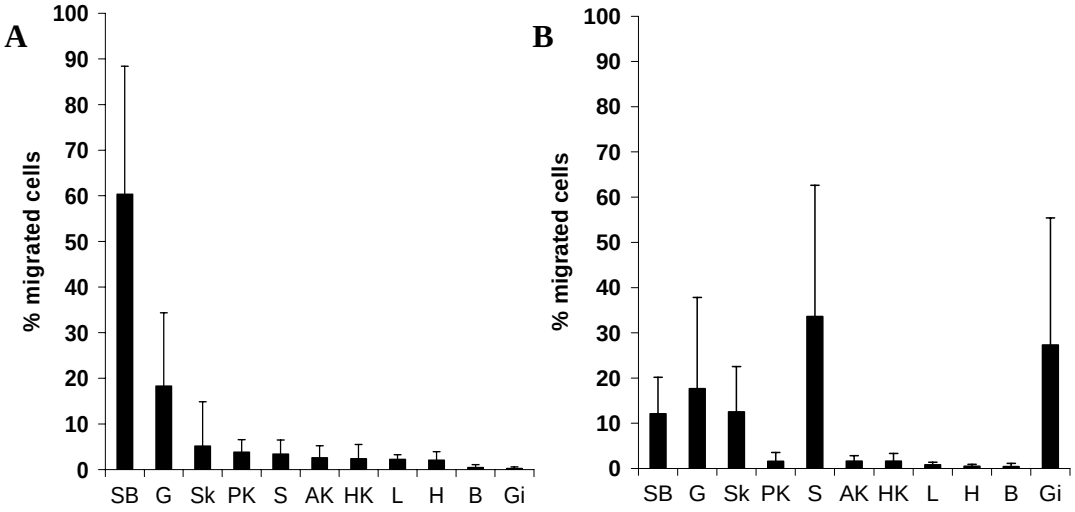

FIGURE 3.9. DCLCs migrate to mucosal sites *in vivo*. (A) DCLCs were stained with CFSE and injected intraperitoneally into recipient fish. Twenty-four hours later, flow cytometry was used to determine the number of CFSE-positive events in 1 million collected events per tissue. The number of CFSE-positive events in all tissues was totaled and events per tissue were divided by this total to arrive at percent of migrating cells in each tissue. DCLCs migrate primarily to mucosal sites (A); however, after TLR-ligand treatment this pattern shifts. (B) DCLCs were exposed to TLR ligands for 24 hours, washed, stained and transferred as in (A). The most striking difference is the trend of activated DCLCs to migrate primarily to spleen. (SB) swim bladder, (G) gut, (Sk) skin, (PK) posterior portion of trunk kidney, (S) spleen, (AK) anterior portion of trunk kidney, (HK) head kidney, (L) liver, (H) heart, (B) blood, (Gi) gills. Data are from four experiments.

The rationale for the approach taken here was that DCs or DC-like cells could be generated from fish tissues using methods similar to those employed for the production of bone-marrow derived DCs in mammals. Typically, this involves the addition of growth factors to bone marrow cells. Initially, we added recombinant human GM-CSF to trout cell cultures but found that we could generate DCLCs even in its absence. This is similar to long-term stromal cell cultures from mouse spleen that

support the production of immature DCs without the requirement for GM-CSF (18). We therefore presume trout DCLCs emerge from progenitor cells under the influence of cytokines being produced in culture in an autocrine or paracrine fashion. As noted in the text, DCLCs appear to develop from non-adherent monocytic cells in culture. However, when these cells were removed and the media replenished, DCLCs continued to emerge for up to 4 months indicating that they may arise from adherent precursors in addition to monocytic progenitors. Depending on the fish from which tissues were excised, there was considerable variation in the number of DCLCs that developed, the time at which they emerged, and the length of time they continued to be produced in culture.

Although the studies reported here describe cultures from head kidney, anterior portion of trunk kidney, and spleen tissues together, trout DCLCs could also be generated from each of those tissues individually, as well as from the posterior portion of the trunk kidney. Larger numbers of melanomacrophages (along with unidentified debris) appeared in cultures containing posterior portions of the trunk kidney, and were therefore avoided. Interestingly, cells with essentially the same morphology as DCLCs could also be generated from peripheral blood monocytes of rainbow trout or isolated from spleen using methods adapted from the mammalian literature (data not shown). Functional studies are being undertaken to determine whether these cells are also capable of antigen-presentation.

DCLCs possess distinctive multi-lobed nuclei that resemble the nuclei of polymorphonuclear cells (PMNs). PMN nuclei; however, are typically circular in arrangement, while DCLCs most often display a clover leaf-type arrangement with multiple lobes linked at a central point by thin extensions of nuclear material. Early

publications describing mammalian DC cultures show cells with strikingly similar nuclear morphology to DCLCs that do not stain with granulocyte antibodies, suggesting that these cells are typical of DC cultures (18, 19). Additionally, DCLCs are not highly phagocytic, as would be expected of PMNs. DCLCs do phagocytose beads, but the cells do not appear to fill to capacity as is typically observed for PMNs and macrophages. The scarcity of granules in DCLCs added to this leads us to conclude that these cells are not PMNs.

While only a limited number of specific antibodies are available for delineating cell types in fish, we show that trout DCLCs fail to bind either IgM or thrombocyte-specific antibodies. By contrast, rabbit antisera against rainbow trout MHC class II bound uniformly to these cells suggesting a role in antigen presentation. In addition to the surface expression of MHC class II, we showed that CD83 and MHC class II genes are expressed in these cells using real-time RT-PCR. CD83, a specific marker for mature DCs in mammals, has recently received a great deal of scrutiny as its function is unraveled. While the full story has not been elucidated it does appear that its expression is correlated with MHC class II surface expression (19). The pattern of CD83 mRNA tissue expression is reported to correlate with that of MHC class II mRNA in rainbow trout, suggesting that, like mammals, CD83 and MHCII expression may be correlated in fish cells (14). CD83 gene expression has been reported in fish leukocytes including trout macrophages (where it is up-regulated in response to LPS)(20, 21), the RTS11 macrophage-like cell line (22), a melanin-producing leukocyte cell line (23), and the Atlantic salmon phagocytic TO cell line (9), as well as endothelial cells (24). While CD83 expression is reported in a range of mammalian cells including T-cells (25), B-cells (26), neutrophils (27), monocytes, and macrophages, stable surface expression is only seen in DCs (28). It is possible that

CD83 mRNA expression is not a specific marker for DCs in fish, however, it should be noted that mRNA levels do not always correlate with surface expression of the translated protein, and CD83 could undergo post-translational regulation in fish as it does in mammals (29). Therefore, despite mRNA expression in cells that most likely are not DCs in fish, surface expression of CD83 protein may still be a specific marker for DCs in teleosts.

Irrespective of the role of CD83 in antigen-presentation, we found that CD83 mRNA transcripts, along with surface levels of MHC class II were up-regulated in trout DCLCs in response to addition of TLR-ligands to culture media. Up-regulation of the expression of surface MHC class II and CD83 mRNA transcripts are clearly consistent with the activation pattern seen in mammalian DCs treated with TLR-ligands. To examine this further, we investigated whether the phagocytic capacity of trout DCLCs was also altered in response to PAMPs. In particular, we were curious to know whether DCLCs became less phagocytic, as mammalian DCs do, following activation via Toll-like receptor signaling. Instead, we found that phagocytosis in trout DCLCs increased in some cases and decreased in others. While the reasons for this are unclear, differences in the maturation state of DCLCs from culture to culture could account for the inconsistent response. Relatively immature cells could become more phagocytic with the addition of TLR ligands, taking longer to reach a mature state where they would lose their phagocytic capacity. On the other hand, cells that are relatively mature may be highly phagocytic and become less so with the addition of TLR-ligands. The maturation state of any given culture could depend on the relative levels of endogenous cytokines present, which in turn may vary from culture to culture. As with mammalian DCs, the relative maturation state of trout DCLCs could

potentially be normalized by the addition of exogenous cytokines to cell cultures, or by treatment with TLR-ligands such as CpG (30).

The defining characteristic of DCs is their superior ability to present antigen to naïve T-cells. Here we show that trout DCLCs induce potent proliferative responses in the primary MLR and are far better in this respect than either B-cells or macrophages. To our knowledge, this the first direct evidence that specialized antigen-presenting cells exist in fish, and while it remains to be determined whether trout DCLCs are homologs to mammalian DCs, their effectiveness in presenting antigens would suggest that they can bridge innate and adaptive immunity in trout. This role is further supported by the migration of transferred DCLCs to mucosal sites in recipient animals, much as immature DCs/Langerhans cell precursors migrate to mucosal sites in mammals, where they take up residence. It is from these outposts that DCs detect microbial products and become activated. Interestingly, Koppang et al found that expression of MHC class II genes in Atlantic salmon was highest in mucosal tissue (31). Together with the data presented here, this could point to a central role for mucosal sites in fish immunity.

While the relationship between trout DCLCs and mammalian dendritic cells is open to interpretation, certain fundamental characteristics would be expected to be conserved in specialized APCs. The unique efficacy of DCs to activate naïve T cells, as well as induce tolerance and anergy, is attributed to their high levels or lack of costimulatory molecules, respectively. Genes encoding costimulatory molecules are beginning to be identified in fish; the expression patterns, as to cell type, have yet to be explored. As the sequences for these molecules become available, their expression in DCLCs can be determined to address further the role of these cells in mounting an adaptive response.

Finally, aside from its inherent biological significance, the identity of cells responsible for antigen presentation in fish has enormous practical importance as well. Fish represent the single most important source of dietary protein for a large part of the world's population, and while demand grows apace, precipitous declines in ocean fisheries have put ever increasing demands on aquaculture as a source of food. Fish raised under intensive farming conditions are highly susceptible to infectious disease. With losses estimated at 10-50% in typical aquaculture settings, infectious diseases clearly have a significant impact on the ability to meet this demand (32). Such losses could be greatly minimized through immunization, and while vaccines are available for a number of fish pathogens, their efficacy varies (32, 33). In addition to on-going work to identify new adjuvants acting through Toll-like and other pattern-recognition receptors, direct targeting of antigen peptides to DCs (through linkage to DC-specific antibodies) has proven to elicit T-cell response that are highly superior to traditional antigen and adjuvant vaccines (34, 35). Clearly such approaches have relevance to the targeting of vaccines in fish, but only if the functional equivalent of DCs can be identified and characterized in these animals.

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CHAPTER 4

Discussion

1. Summary of Findings

While it is generally accepted that antigen presentation occurs in fish, and in a manner similar to that in mammals, the cells principally involved have not been identified. While B-cells and monocytes/macrophages are capable of antigen presentation in fish, DCs, the most potent APC and the only one able to initiate primary adaptive responses in mammals, have not been definitively described. Some evidence for their existence, almost exclusively based on morphology, is reported, but no functional studies have been done. Because of their unique role in bridging innate and adaptive immunity, DCs are of particular interest in fish, the most ancient vertebrates to possess both innate and adaptive immunity.

In order to identify DCs or their equivalent in fish, I developed methods to culture presumptive DCs for further characterization. In chapter 2, I describe several methods that were successful in generating cells with dendritic morphology. The first was based on mammalian peripheral blood monocyte derived DC protocols, and involved culturing the adherent cells from the monocyte fraction of whole blood. The second was modeled after mammalian bone marrow derived cultures, wherein trout hematopoietic tissue (spleen and kidney) was cultured. The last method used was the original protocol for isolation of DCs from spleen, and was used essentially unmodified. While all these methods yield cells with dendritic morphology, the hematopoietic cultures yielded the largest numbers of cells which could then be submitted to various *in vitro* assays.

Characterization of these hematopoietic tissue-derived cells is presented in chapter 3. Morphologically, the cells were a homogenous population and did not appear to be granulocytes, monocytes/macrophages, or lymphocytes by light microscopy. They

expressed surface MHC class II, and transcripts for the mammalian DC marker, CD83. The cells were activated by exposure to TLR-ligands, and were phagocytic. These cells proved to be capable of stimulating primary responses, as assessed by the primary MLR, and were more effective at stimulating division of responders, in that assay, than B-cells or macrophages when compared side by side. Given their efficacy at stimulating the MLR, we proposed that these cells were specialized antigen presenting cells and could be functional equivalents of mammalian DCs. To determine if they migrated to putative sites of antigen presentation, we transferred these cells to observe homing *in vivo*. When injected i.p. into recipient fish, DCLCs migrated primarily to mucosal sites, while activated cells homed primarily to spleen.

In summary, I report a homogenous cell type that can be cultured by methods similar to those used in mammals to generate DCs. These cells resemble mammalian DCs in morphology and function. The most salient similarity to DCs is the superior ability to stimulate the MLR. I suggest that this cell type may act as a link between the innate and adaptive immune systems in fish, as DCs do in mammals.

2. Future Directions and Unanswered Questions

While the results I describe here are compelling, they beget more questions than they answer. This is the beginning of story that is likely to be long and complex. In an attempt to avoid some of the pitfalls currently faced by mammalian immunology, namely the morass of cell surface markers (sometimes of unknown function) required to identify specific cells, the focus in characterizing fish cells should be on functional assays. First, full characterization of the DCLCs from spleen and peripheral blood using the same assays as used for the DCLCs from hematopoietic cultures should be carried out to establish if these cells are functional equivalents. If functional

equivalency is found, then cells could be used interchangeably depending on the requirements of the experiment.

Next, the examination of the antigen presentation function of DCLCs in more depth would be indicated. While I suspect that DCLCs are capable of stimulating an antigen specific response, given their efficiency in the MLR, concrete evidence is needed. Syngeneic fish could be vaccinated with a model protein, or ideally a relevant pathogen-associated protein. Primed Ig- lymphocytes could then be isolated from spleen (or peripheral blood) and used as responders with autologous DCLCs pulsed with protein antigen as stimulators. DCLCs not exposed to antigen could be used as controls. Once this has been demonstrated, a larger related question can be tackled: can DCLCs initiate a primary response *in vivo*? It has been shown that DCs pulsed with antigen can prime T-cells when transferred to naïve mice (1). A similar experimental approach could be used in fish. DCLCs pulsed with antigen could be used to vaccinate MHC matched or mismatched recipient fish to prime T-cells in the animal. Spleen Ig- lymphocytes (or PBLs) could be isolated and secondary responses to MHC matched or mismatched antigen pulsed DCLCs could be examined to determine if an antigen specific response was initiated in the animal. DCLCs not pulsed with antigen or pulsed with a heterologous antigen could serve as controls. It is difficult to determine an appropriate control for this experiment as other cell types could potentially present the protein antigen obtained from the pulsed DCLCs *in vivo*. The use of mismatched MHCs is one approach to control for this problem. In order to examine their role *in vivo* further, a disease model would be extremely useful.

As discussed in Chapter 3, the maturation state of the DCLCs is unknown and may vary from culture to culture. In humans CpG exposure has been used to mature

peripheral blood DC populations resulting in greater T-cell stimulation (2). A preliminary experiment conducted showed increased CD83 and MHC class II mRNA expression in DCLCs treated with CpG, GM-CSF, or GM-CSF+IL-4 for 4 days compared to media controls (Fig. A.4), suggesting that a similar treatment may be useful in generating a mature DCLC population. Alternatively, maturation of DCLCs could be attempted in context and an immunostimulant, such as CpG, could be added directly to cultures prior to harvest. Phagocytosis assays and MLRs could then be carried out to assess how these treatments affect function.

In light of recent data that suggest MHC class II expression is more widespread in fish than mammals (3), expression of MHC class II alone may not indicate APC function in fish. Expression of costimulatory molecules in addition to MHC class II may be necessary to determine what cell types can function as APCs. A partial sequence for a CD80/86-like molecule has recently been identified in Atlantic salmon which is likely to be useful in trout as well (4). Once it is established that costimulatory molecule expression levels correlate with capacity to stimulate T-cells in fish, then expression of costimulatory molecules could be used to predict the potential of cell types to act as APCs. Various treatments and their effects on costimulatory molecule expression could be explored. Identification of adjuvants that induce high levels of costimulatory molecules would be useful in vaccine preparations and experimental design.

Since we propose that DCLCs are a specialized antigen presenting cell, it stands to reason that these cells will express genes that are involved in antigen sensing, uptake, processing and presentation. In order to look on a larger scale for genes involved (both identified and as yet unidentified), RNA could be isolated from DCLCs, in an un-stimulated state and after exposure to TLR-ligands, and submitted to microarray

analysis. The consortium for Genomic Research on All Salmon Project (cGRASP), which has offered a 16k salmonid microarray since 2004, has completed a 32k cDNA microarray, and a 22k 70mer oligo array that are now available to researchers (5). With the use of these tools expected (known) gene homolog expression could be confirmed and new genes, not yet identified in fish could potentially be found. On a less grand scale expression of genes associated with macrophage function such as natural resistance macrophage associated protein (NRAMP) and M-CSFR could be determined in DCLCs. This could shed light on the identity of these cells and their relationship to monocyte derived cell types. Some subsets of DCs in mice express CD8, however this is not found to be the case in humans. A recent study in Fugu using a CD8α antiserum surmised that CD8α is expressed in monocytes/macrophages in Fugu (6), suggesting there might be some evolutionary significance to this expression pattern. If rainbow trout DCLCs were also found to express CD8α, it would confirm a more evolutionarily widespread pattern of CD8α expression.

A large gap in our understanding that one could tackle is the site(s) of antigen presentation in fish. A more sensitive assay for migration would potentially be to radio-label DCLCs before transfer and directly measure radioactivity in undisrupted organs/tissues after transfer. Using this assay detection of migrated cells should be more straight-forward and have more power statistically, as it allows collection of data from the whole fish rather than a limited number of cells from each tissue. Observation of migration patterns of activated DCLCs versus non-activated DCLCs could then be carried out with this more sensitive method. If it was observed that migration patterns of activated DCLCs differed from non-activated DCLCs, then preferred destinations of activated cells would be good candidates for sites of interaction with naïve T-cells. Another approach, based on the assumption that APCs

reside in the skin, is to paint the skin with FITC-dextran. This would allow one to determine the destination of cells initially located in the periphery (skin) that take up antigens through lectins and migrate—characteristic activities of mammalian DCs. The FITC labeled cells could be located in tissue sections with an anti-FITC antibody or by flow cytometry in single cell suspensions. The sites of antigen presentation and patterns of migration could be applied to vaccine technology, particularly the mode of delivery. The site of vaccine delivery could be tailored to maximize availability of antigen to APCs and subsequent immune responses.

I have conducted preliminary experiments to compare the effects of different TLR-ligands on DCLCs; however, the results are not conclusive (Fig. A.1). Real time RT-PCR could be used to determine which TLRs are expressed in these cells. The pattern of expression would be of comparative value with mammals, but also it will help determine which TLR-ligands will be active on the cells. Particularly, if they express a specific subset of TLRs, DCLCs could prove to be a useful model for studying TLR biology in teleosts (i.e. TLR regulation, signaling, and specificity).

To address the question of whether the existence of a specialized antigen presenting cell is specific to rainbow trout or if they are present in a wider array of teleosts, catfish could be used as an additional teleost model. Catfish are well studied immunologically and a number of useful reagents are available, including the recent publication of an MHC class II monoclonal antibody (3). The protocols used in rainbow trout to generate DCLCs could be applied to catfish to ascertain whether DCLCs can be cultured in other fish by the same methods. The generation of a specialized antigen presenting cell in catfish as well as rainbow trout would indicate broader significance of such a cell type in teleost species.

Using zebrafish, a genetically tractable teleost model, transgene technology has been used to express fluorescent proteins under cell specific promoters allowing real time visualization of cells *in vivo*. One example of this is zebrafish that express GFP under a T-cell specific promoter, the tyrosine kinase lck (7). Particularly in optically transparent larva, T-cells could be seen populating the thymus, and traveling through the circulation. This system has great potential for the study of immunology. The complexity of cell interactions *in vivo* are becoming increasingly apparent and models that allow visualization without *ex vivo* manipulation of the cells, which is sure to modulate cell behavior, would be truly powerful. I began my dissertation research working with zebrafish. I spent a year making promoter constructs and learning zebrafish husbandry under the tutelage of Thomas Paul. CD209, a C-type lectin expressed on DCs, and CD83, a marker for mature DCs in mammals, had both been identified in zebrafish at the start of the project. Using the zebrafish genome, I cloned a region upstream from the CD83 gene start codon and placed it in a vector with the expression of RFP under its control. These putative promoter constructs were then injected into zebrafish embryos in conjunction with the lck-GFP promoter construct. The goal was to generate double transgenic fish that possessed green fluorescent T-cells and red fluorescent putative DCs. I did not progress to the point of generating good candidate transgenic founders nor was I able to clone a CD209 putative promoter region before the relative success of the rainbow trout studies took me away from zebrafish. The potential power of this approach, however, merits further pursuit of this project. Two caveats to this approach are the long term nature of the project (with no sure outcome) and the lack of knowledge of the zebrafish promoters for these genes. If this project were to be resurrected, it would be wise to start with *in vitro* reporter experiments demonstrating specific activity of the putative promoters before injection

of constructs is attempted. Several new assemblies of the genome have been compiled since this project was started; it is likely that more accurate genomic information is now available as well as improvements in methods (such as the use of bacterial artificial chromosomes) that would increase the likelihood of success.

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APPENDIX

Preliminary Experiments

The following represents preliminary experiments carried out relevant to the topic of this dissertation.

1. DCLC Maturation

From experiments in Chapter 3, it is clear that DCLCs respond to TLR-ligands. In order to determine which TLR-ligands were most effective in activating DCLCs, treatment with individual TLR-ligands was carried out. DCLCs were incubated with each TLR-ligand, or the mixture of TLR-ligands, for two to four days and surface expression of MHC class II was examined by flow cytometry. As seen with other experiments, the mixture of TLR-ligands (ssRNA, imiquimod, flagellin, and polyI:C) resulted in an increase in MHC class II expression at the four day time-point (Fig. A.1). As for the individual ligands, these appeared to be more active a day earlier than mixed ligands, although none were as potent as the mixture of ligands (Fig. A.1). From this one experiment, imiquimod and flagellin appear to be the most effective in inducing increased surface expression of MHC class II, but repeat experiments are needed to confirm this. Another point of interest in this experiment is the apparent down-regulation of MHC class II surface expression compared to control at four days in all individual ligands (except LPS), followed by a return to control levels at day 5. In contrast, the mixture of TLR-ligands does not go below control levels at any time, but does return to control levels at day five like the individual ligand treatments. The increased expression of MHC class II in response to flagellin suggests that DCLCs express TLR5. Real time RT-PCR could be performed to confirm this is the case.

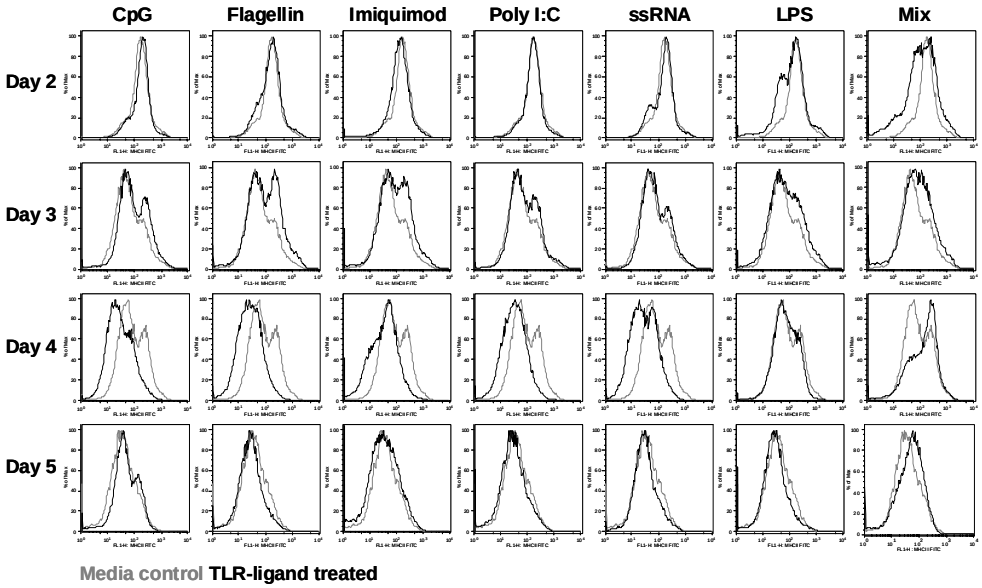

FIGURE A.1. Individual TLR ligand treatment of DCLCs. DCLCs were incubated with individual TLR-ligands or a mixture of ligands for 2, 3, 4, or 5 days and then assayed for MHC class II expression by flow cytometry. 4 day treatment with a mix of TLR-ligands results in up-regulation of MHC class II. Individual TLR-ligands do not appear to have as potent an effect as the mixture. On day 3 flagellin and imiquimod show some increases in expression. Individual ligands appear to act at day 3, followed by decreases in expression on day 4.

Real time RT-PCR was employed to determine the effects of individual TLR-ligand treatment on DCLC CD83 mRNA expression. After 24 hour incubation with the mixture of TLR-ligands, CD83 mRNA expression in DCLCs was increased; however, it appears that only imiquimod and polyI:C may have increased CD83 mRNA levels (Fig. A.2). The large margin of error for Imiquimod and small changes seen with polyI:C make an interpretation difficult. Repeat experiments would help to determine if there are real differences in response to the different ligands at the twenty-four hour time-point. Since the surface expression is seen to be up-regulated at day 3, the observation of mRNA expression levels at forty-eight hours in subsequent experiments may be more informative than at twenty-four hours.

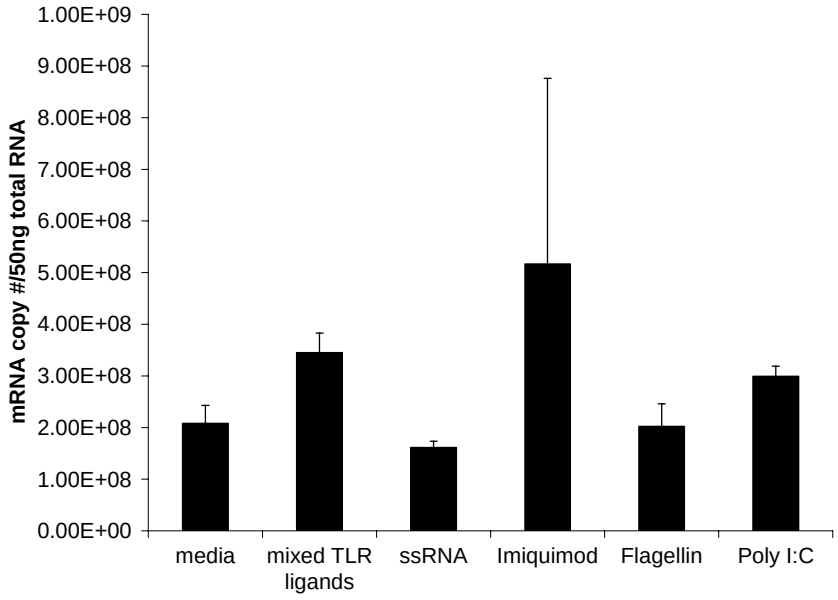

FIGURE A.2. Effects of Individual TLR-ligand treatment on CD83 mRNA expression. DCLCs were exposed to individual TLR-ligands or a mixture of ligands for 24 hours. RNA was extracted from the cells and submitted to real time RT-PCR analysis. Treatment with imiquimod, polyI:C or mixed ligands appears to up-regulate CD83 mRNA expression compared to media control.

As discussed in Chapters 2 and 3, mammalian protocols use cytokines and CpG treatment to generate mature DCs. To begin to examine this, DCLCs were treated for four days with GM-CSF, GM-CSF+IL-4, or CpG, after which CD83 and MHC class II mRNA expression levels were determined. Incubation with GM-CSF and GM-CSF+IL-4 resulted in significant increases of both CD83 and MHC class II mRNA copy numbers above controls ($p<0.05$) (Fig. A.3). CpG treatment induced significant up-regulation of MHC class II mRNA copy numbers, but not CD83 mRNA copy numbers. This suggests that cytokines are more efficient at inducing maturation of DCLCs, at least using MHC class II and CD83 mRNA expression as a measure of maturation. The ability to stimulate the MLR may be a better indicator of maturation than mRNA expression levels.

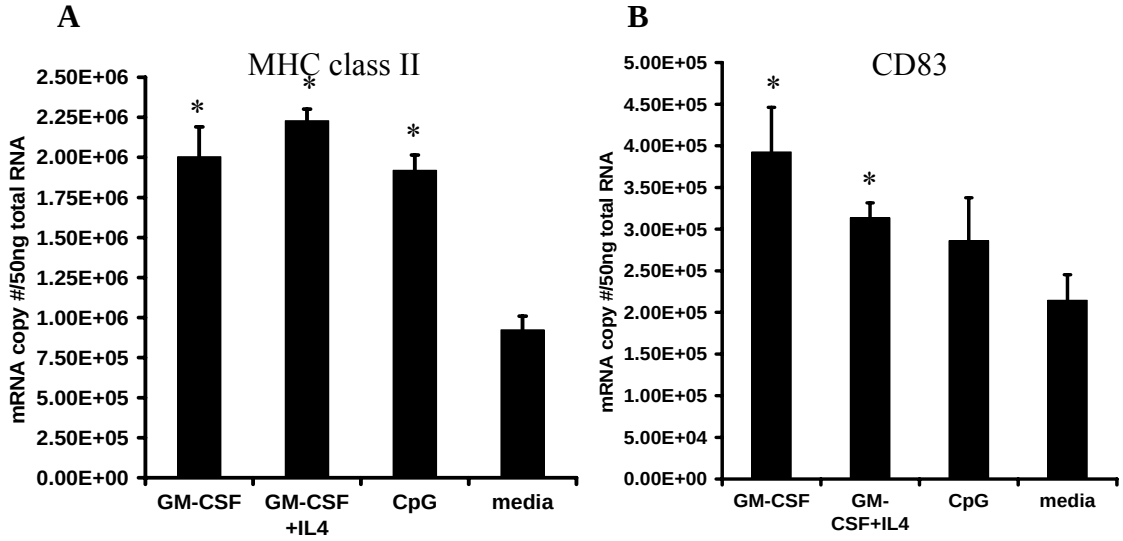

FIGURE A.3. Maturation of DCLCs. DCLCs were cultured in the presence of GM-CSF, GM-CSF+IL-4, or CpG for four days. RNA was then extracted from the cells and submitted to real time RT-PCR analysis. A significant increase in MHC class II mRNA compared to control (denoted by (*), $p<0.05$) was seen in cells treated with GM-CSF, GM-CSF+IL-4, and CpG (A); however, for CD83 mRNA, significant increases were seen only in GM-CSF and GM-CSF+IL-4 treated cells (B) ($p<0.05$). Further experiments would be necessary to determine if CD83 or MHC class II mRNA expression best reflects maturation of cells.

2. Comparison of DCLCs to Other Cell Types

Since there are few definitive leukocyte markers in fish, cell type can be determined through comparison with functionally defined cell types. To begin this comparative analysis I examined CD83 mRNA expression levels in DCLCs and in fish tissues, including those tissues from which DCLC cultures are derived. When compared to spleen, head kidney, and kidney DCLCs are enriched for CD83 transcripts, suggesting that CD83 expression in these tissues may stem primarily from DCLCs present (Fig. A.4A). Taking the lack of reagents into account, another approach to comparative analysis is FACS cell sorting of distinct populations based on forward/side scatter. When CD83 mRNA expression levels of sorted lymphocyte and granulocyte populations are compared to expression levels in DCLCs, DCLCs are highly enriched for CD83 mRNA transcripts (Fig. A.4B).

Perhaps the most telling comparison of DCLCs is to macrophages. Because of the similarity in culture methods described for generation of macrophages in fish and the general assumption that monocytes/macrophages are the main APCs in fish, I directly compared these cell types. DCLCs were compared to resting peritoneal macrophages in terms of MHC class II expression, morphology, and phagocytosis. DCLCs express higher levels of MHC class II than macrophages (Fig. A.5A and B). And, as can be seen in figure 3.4, macrophages are round adherent cells, while DCLCs are non-adherent and irregular in shape. When mixed with 1μm beads macrophages avidly phagocytose beads to the point that their cytoplasmic space is filled to capacity with beads (Fig. A.5E), while DCLCs have many fewer beads per cell (Fig. A.5F).

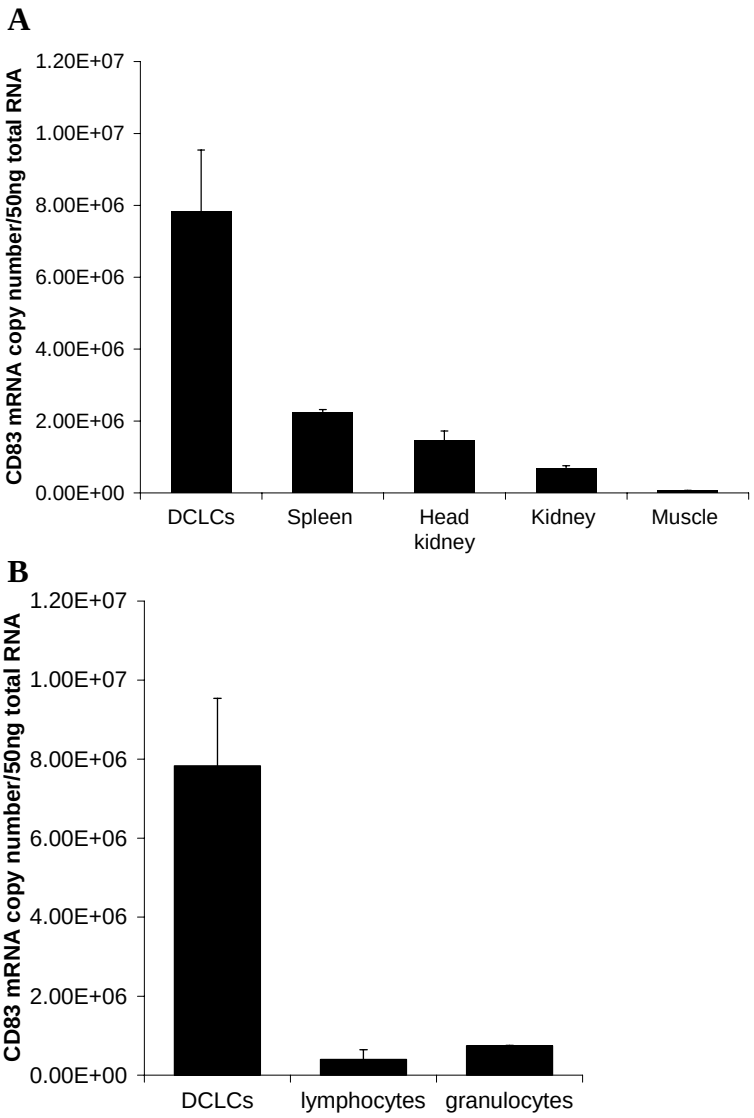

FIGURE A.4. CD83 expression. DCLC CD83 mRNA expression was compared to fish tissues. DCLCs, lymphocytes, and granulocytes were sorted based on forward/side scatter profiles. RNA was extracted from the separate cell populations and real time RT-PCR was performed. DCLCs express higher levels of CD83 mRNA compared to either sorted lymphocytes or granulocytes.

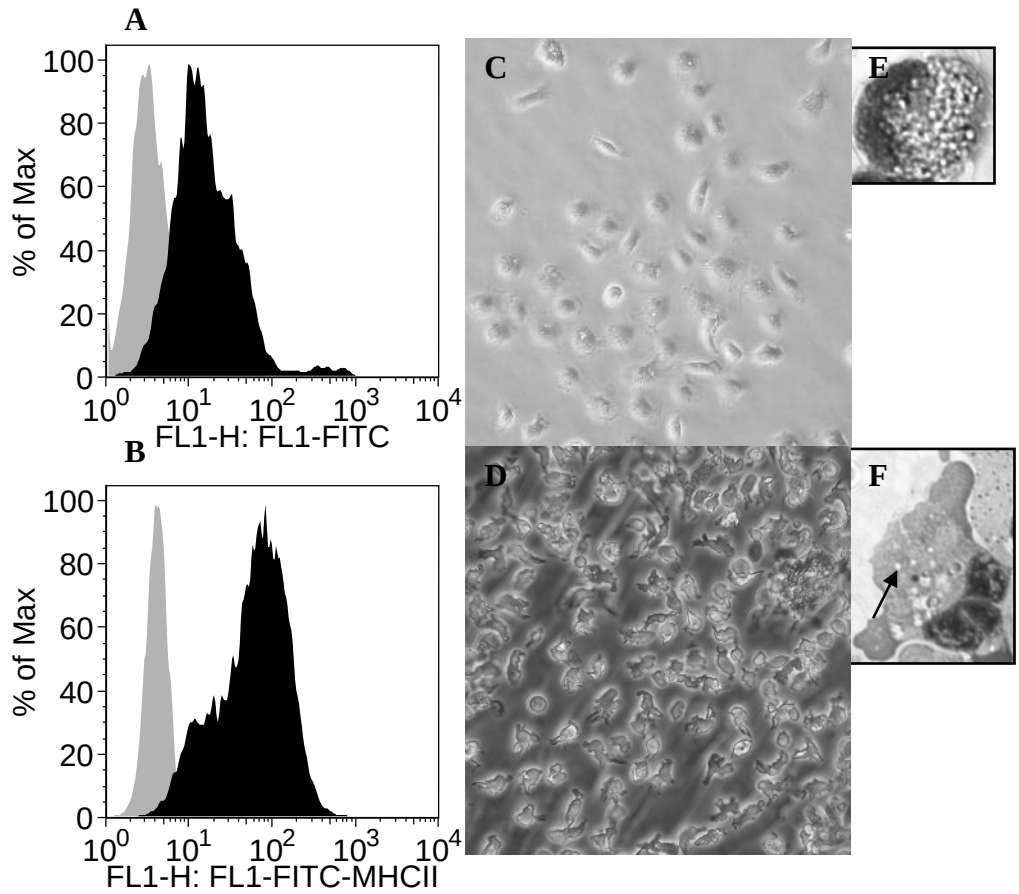

FIGURE A.5. Comparison of hematopoietic DCLCs to macrophages. (A) MHC classII expression of resident peritoneal macrophages (black filled histograms, grey histograms are pre-immune serum). (B) DCLC MHC class II staining (black histograms, grey histograms are staining with pre-immune serum). Phase contrast of peritoneal macrophages (C), showing rounded adherent cells, compared to DCLCs (D), which are non-adherent and dendritic in morphology. Peritoneal macrophages take up large numbers of beads per cell (E), cell is full of beads compared to DCLCs (F), which are phagocytic, but do not take up large numbers of beads per cell.